VOLUME 32 JULY 1954 NUMBER 4

Canadian Journal of Biochemistry and Physiology

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF BIOCHEMISTRY AND PHYSIOLOGY

(Formerly Canadian Journal of Medical Sciences)

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Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 32 JULY 1954 NUMBER 4

THE LACK OF EFFECT OF VITAMIN E ON THE BLOOD CLOTTING MECHANISM[1]

BY RACHEL M. PAUL[2], J. A. LEWIS[3], AND H. A. DELUCA[4]

Abstract

The oral administration of vitamin E to human subjects in daily doses of 600 mgm. for seven days failed to evoke any blood change in clotting times, prothrombin times, or plasma fibrinogen levels. There was a small increase in platelet level which was statistically of borderline significance.

There have been several reports that suggested that vitamin E may inhibit the blood-clotting mechanism both *in vivo* and *in vitro*. Zierler *et al.* (12) reported that the clotting time of plasma taken from rats previously given vitamin E was prolonged; they also indicated that the clotting time of plasma could be lengthened by the addition of this agent *in vitro*. From their work, they concluded that vitamin E acts as an antithrombin. In a series of papers (1, 2, 3) Kay and his collaborators came to similar conclusions, although their experimental procedures were somewhat different.

The present report includes the results of an investigation undertaken to determine what effect the administration of vitamin E has on the clotting properties of blood taken from subjects who have received relatively large doses of vitamin E over a period of seven days. The effect on the clotting mechanism was investigated by determining (1) clotting times, (2) prothrombin times, (3) platelet counts, and (4) fibrinogen levels. Plasma vitamin E levels were carried out concurrently. Each estimation was performed by the same operator at a given time each day.

Experimental Procedure

A group of 11 male patients was selected from the medical wards of Westminster Hospital in London. None of these subjects had an illness nor was receiving therapy that might presumably affect the blood-clotting mechanism. The five estimations indicated above were carried out on each of these patients for three consecutive days. This period has been called the

[1] *Manuscript received November 20, 1953.*
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"control period". Six of these patients were then fed daily 600 mgm. of vitamin E in the form of alpha-tocopheryl acetate for seven days; the remaining five patients were given matching placebos (cocoa) for the same period. This experimental period has been called the "test period". The five estimations mentioned above were carried out on the fifth, sixth, and seventh days of the test period.

Methods

Clotting Times

These were determined in both glass and silicone-treated tubes. The method of Lee and White (4) was used for the glass-tube clotting times; that of Margulies and Barker (5) for the silicone tubes. Estimations were performed in duplicate in each instance.

Prothrombin Times

The method of Quick (8) was used for the determination of prothrombin time. Estimations were carried out in triplicate.

Platelet Counts

The procedure as outlined by Moolton and Vroman (6) was followed for the estimation of platelet count. Duplicate determinations were performed on each sample.

Fibrinogen

The fibrinogen concentration was determined by weighing the fibrin clot formed from 1 cc. plasma. The plasma sample was allowed to stand at room temperature until there was complete clot formation. The clot was then wound on a glass rod, rinsed well with distilled water, dried on a filter paper, and placed in a previously weighed vial. It was next dried over phosphorus pentoxide for a minimum of three days and then weighed.

Vitamin E

The vitamin E levels in the plasma were estimated in duplicate by the method of Quaife *et al.* (7).

Results

In Table I are shown the mean values for the various estimations obtained during the control and test periods for the placebo and vitamin E groups respectively. In Table II the results are expressed as mean "test percentages". The test percentage has been defined as:

$$\frac{\text{value of a given estimation during test period}}{\text{value of the same estimation during control period}} \times 100 .$$

This index has been determined for the estimation on each subject in the placebo and vitamin E groups. A mean test percentage greater than 100 indicates a mean increase in a given value obtained during the test period compared with the control period.

TABLE I

MEAN VALUES FOR ESTIMATIONS IN THE CONTROL AND TEST PERIODS

	Clotting time, min.		Prothrombin time, %	Platelet count in thousands per cu. mm.	Fibrinogen, mgm. %	Vitamin E, mgm. %
	Glass tube	Silicone tube				
	Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.
Placebo group						
Control	11.2 ± 0.3	49.3 ± 4.9	91.9 ± 2.9	260.2 ± 15.1	465 ± 21	0.78 ± 0.03
Test	13.0 ± 0.5	60.8 ± 6.8	91.1 ± 2.6	255.0 ± 26.0	468 ± 17	0.76 ± 0.04
Vitamin E group						
Control	11.1 ± 0.3	47.4 ± 2.6	88.7 ± 3.8	235.2 ± 11.0	482 ± 21	0.87 ± 0.09
Test	12.1 ± 0.4	52.0 ± 3.2	92.1 ± 2.9	273.8 ± 16.4	463 ± 19	1.55 ± 0.19

TABLE II

MEAN TEST PERCENTAGES

	Clotting time		Prothrombin time	Platelet count	Fibrinogen	Vitamin E
	Glass tube	Silicone tube				
	Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.
Placebo group	116.6 ± 4.7	123.0 ± 4.2	100.0 ± 4.6	97.4 ± 6.0	102.8 ± 6.3	98.6 ± 2.7
Vitamin E group	108.0 ± 5.6	110.0 ± 6.1	104.6 ± 3.3	116.0 ± 5.3	96.5 ± 5.2	177.8 ± 10.8
P value	> 0.4	> 0.1	> 0.3	0.05	> 0.4	< 0.01

Discussion

Except for the elevated fibrinogen levels, the values obtained during the control period for both the placebo and vitamin E groups did not differ significantly from values which had previously been determined in this laboratory on a group of healthy males.

Clotting Times

Table II shows that there was a slight increase in the glass-tube clotting times in the placebo group during the test period as compared with the control period as is indicated by a mean test percentage of 116.6. There was a similar but smaller increase in the mean test percentage for the vitamin E group (108.0). Analyzed statistically, however, it is evident that no change in clotting time was obtained in these experiments from the administration of the vitamin E ($P > 0.4$). The same conclusion is reached from a consideration of the data obtained by the use of silicon-treated tubes.

As already indicated, the vitamin E was given for seven days in daily doses of 600 mgm. For the average subject this dose represents slightly less than 0.01 gm. per kilogram body weight. In the experiments of Zierler *et al.* (12), the rats were given single intraperitoneal injections of vitamin E (disodium salt of α-tocopheryl phosphate) in doses ranging from 0.01 to 2.0 gm. per kilogram body weight. Blood samples were taken 30 min. later for blood-clotting determinations. A critical analysis of their results indicates that no significant change in clotting time occurred until the vitamin E dosage reached 0.1 gm. per kilogram body weight. This is at least ten times larger than the daily dose given the subjects in the present investigation. Inasmuch as the dose chosen in this work is equal to the maximal amount (600 mgm.) usually given when vitamin E is used for therapeutic purposes, it would appear that this amount is insufficient to produce any change in clotting time.

Prothrombin Times

The mean test percentages given in Table II indicate that there was no change in prothrombin attributable to the administration of vitamin E.

Fibrinogen Levels

As already indicated, the fibrinogen levels during the control period for both the placebo and vitamin E groups (465 and 482 mgm. % respectively) were higher than those previously obtained in this laboratory for a group of healthy males (271.0 $\pm$ S.D. 33.1). This latter value is in agreement with the range generally given for normal individuals. The reason for the elevated fibrinogen levels is not clear. The only obvious difference between the "normal" and "experimental" groups lies in the fact that the former was composed of young healthy males while the latter group consisted of older male subjects who were hospitalized. In any event, there was no change in fibrinogen levels as a result of the administration of vitamin E (Table II).

Platelet Levels

Table II indicates that the platelet level remained constant within experimental limits during the control and test periods in the placebo group as shown by a test percentage of 97.4. On the other hand, the platelet level rose to 116.0% during the test period in the vitamin-treated group. These results suggest that this slight increase in platelets is a result of vitamin E administration but it is statistically only of borderline significance ($P = 0.05$).

TABLE III

THE EFFECT OF VITAMIN E ON PLATELET COUNT

	Subject	Platelets in thousands per cu. mm.		
		Control	Test	Difference
Placebo group	1	260	206	− 54
	2	241	217	− 24
	3	216	223	+ 7
	4	302	345	+ 43
	5	282	284	+ 2
	Percentage day-to-day variation (Mean ± S.E.M.)	8.4 ± 2.2	9.1 ± 4.2	
Vitamin E group	1	217	228	+ 11
	2	268	306	+ 38
	3	205	211	+ 6
	4	337	443	+106
	5	226	247	+ 21
	6	158	208	+ 50
	Percentage day-to-day variation (Mean ± S.E.M.)	5.1 ± 1.0	6.8 ± 1.8	

It might be of interest to examine the mean values for the platelet levels of the individual subjects. These are shown in Table III. As is well known, the platelet level for a given individual over a period of time can be established only within fairly broad limits. Duplicate counts on a blood sample may be made with moderate precision. In the present investigation, the average percentage deviation of the duplicate mean was 4.6 ± S.D. 3.4. Of more consequence, however, is the variation from day to day in the platelet count itself. In a thorough investigation of the subject under ideal conditions, Sloan (11) has shown that the day-to-day count may vary considerably. The present investigation would tend to confirm this. In Table III is shown the

mean percentage day-to-day variation for each of the four groups. If there is an actual increase in platelet level as a result of administration of vitamin E under the conditions of this investigation, it is obviously small and tends to be masked by the day-to-day variations.

An increase in platelets as a result of vitamin E administration for a period of 7 to 14 days in several types of purpuras has been reported by Skelton and co-workers (10). It is possible that significant increases in platelet levels might have been obtained in the present investigation following a longer period of vitamin E administration. While such indices as clotting time or prothrombin time could conceivably change in such a short period (seven days) as a result of the marked increase in the tocopherol content of the plasma, a large change in platelet level would hardly be expected.

An investigation similar to the present one has been reported recently by Salvini and Scardigli (9). These workers gave intramuscular injections of 100 mgm. of vitamin E daily to normal subjects over a 10-day period. At the end of this time they found marked increases in fibrinogen levels (up to 71%) and prothrombin activities (up to 28%) but no change in platelet levels nor in clotting times. Their findings (except for the clotting times) are not in agreement with those reported in the present communication. The failure to find an increased platelet level as a result of vitamin E administration is understandable but it is difficult to reconcile their findings relative to fibrinogen and prothrombin levels with those reported here.

References

1. KAY, J. H., HUTTON, S. B., and WEISS, G. N. Bull. Tulane Med. Fac. 9 : 122. 1950.
2. KAY, J. H., HUTTON, S. B., WEISS, G. N., and OCHSNER, A. Surgery, 28 : 24. 1950.
3. KAY, J. H. Yale J. Biol. and Med. 23 : 515. 1951.
4. LEE, R. I. and WHITE, P. D. Am. J. Med. Sci. 145 : 495. 1913.
5. MARGULIES, H. and BARKER, N. W. Am. J. Med. Sci. 217 : 42. 1949.
6. MOOLTON, S. E. and VROMAN, L. Am. J. Clin. Pathol. 19 : 701. 1949.
7. QUAIFE, M. L., SCRIMSHAW, H. S., and LOWRY, O. H. J. Biol. Chem. 180 : 1229. 1949.
8. QUICK, A. J. *In* Blood clotting and allied problems. *Edited by* J. E. Flynn. Transactions of the first Macy Conference, New York. 1948.
9. SALVINI, L. and SCARDIGLI, G. Clin. Therap. 2 : 357. 1952.
10. SKELTON, F., SHUTE, E. V., SKINNER, H. G., and WAUD, R. A. Science, 103 : 762. 1946.
11. SLOAN, A. W. J. Clin. Pathol. 4 : 37. 1951.
12. ZIERLER, K. L., GROB, D., and LILIENTHAL, J. L. Am. J. Physiol. 153 : 127. 1948.

SUBCUTANEOUS FAT AND SKIN TEMPERATURE[1]

By Jacques LeBlanc

Abstract

The subcutaneous fat explains to a large extent the individual differences, as well as the regional variations in the skin temperature, observed on the body. The insulation of the fat layer is 0.41 Clo per inch after one hour's exposure at 70° F., 1.18 at 60° F., and 1.96 at 50° F. The significance of these results, with reference to the thermal steady state of the body, is discussed.

Introduction

Because of the high insulating properties of fat, it may be postulated that in a cold environment the heat loss is smaller in fat than in thin individuals. Some workers assumed that the lower skin temperature observed in women exposed to cold was due to the fact that they had more subcutaneous fat than men (5). It has been shown also in nonliving tissues that the thermal conductivity of muscles is twice that of fat tissues (6).

Aside from theoretical estimations and *in vitro* observations of the insulating value of fat, not much work has been done on the effect of subcutaneous fat in men exposed to cold. Consequently, a systematic study at different environmental temperatures on fat and lean persons, with known individual subcutaneous fat thickness, would be helpful in our comprehension of the physiological effects of cold and would also prove useful in the interpretation of experiments done in cold rooms or in the field.

Protocol

Six young males from our laboratories were used as subjects. They sat quietly, with their arms away from the body, for periods of one hour in a still air room at temperatures of 50°, 60°, and 70° F. Skin temperatures were taken at 20 places over the trunk after 20, 40, and 60 min. of exposure. A thermocouple, made of 36 gauge copper and constantan wires and connected to a Brown potentiometer, was used for these measurements which could be made in 1½–2 min. (4). The subcutaneous fat thickness was evaluated by holding a fold of skin and measuring it with a caliper (2); the results obtained with this procedure were then divided by two. These estimations were made at 20 places on the trunk.

Results

The individual weight and height and the subcutaneous fat thickness of the trunk are reported in Table I. The average skin temperatures, after 20, 40, and 60 min. at 50°, 60°, and 70° F., are given in Table II.

As expected, the fall in the skin temperature of all the subjects is larger during the first 20 min. of exposure than during the second or third. Also, the rate of fall is affected by the environmental temperature; it is faster at 70° F. than at 60° F. and faster at 60° F. than at 50° F.

[1] *Manuscript received December 22, 1953.*
Contribution from Defence Research Medical Laboratories, Toronto, Ontario.

TABLE I

INDIVIDUAL CHARACTERISTICS

Subject	Weight (lb.)	Height (in.)	Chest diam. (in.)	Av. subcutaneous thickness of trunk (mm.)
D.C.	129	69.5	35	2.25
G.S.	146	72.5	38	3.25
A.D.	149	66.25	36	5.25
J.L.	156	66.25	39	6.00
G.M.	148	65.75	37	8.50
F.R.	175	64.25	40	11.00

TABLE II

EFFECT OF EXTERNAL TEMPERATURE AND TIME OF EXPOSURE ON THE AVERAGE SKIN TEMPERATURE OF THE TRUNK

	70° F.			60° F.			50° F.		
	20 min.	40 min.	60 min.	20 min.	40 min.	60 min.	20 min.	40 min.	60 min.
D.C.	92.50	92.25	90.25	88.00	87.75	87.75	82.75	83.00	83.00
G.S.	91.50	90.25	89.00	86.75	86.25	86.25	81.50	80.75	80.25
A.D.	90.25	90.00	89.25	86.25	86.25	85.50	80.75	80.25	80.00
J.L.	90.50	90.25	89.50	85.75	83.50	83.50	80.75	79.25	78.25
G.M.	89.00	86.00	86.25	83.50	81.75	81.50	76.75	74.00	73.25
F.R.	90.00	89.00	88.50	81.75	81.25	80.75	75.00	72.75	71.75

An index of the thermal physiological state of the tissue was given by Burton (1). This index is valid only when a thermal steady state has been reached. Since it is mainly affected by changes in the peripheral circulation, it was termed the "thermal circulation index" and is equal to:

$$(T_S - T_E)/(T_R - T_S).$$

$$(T_R - T_S)/(T_S - T_E) = 1.21\ I_T/I_A\ .$$

Where T_E = environmental temperature in ° F.,
T_R = core or rectal temperature in ° F.,
T_S = skin temperature in ° F.
I_T = insulation of the tissues,
and I_A = insulation of the air.
We make $I_T = I_B + I_F$,
where I_B = insulation of tissues other than fat,
and I_F = insulation of the fat layer.

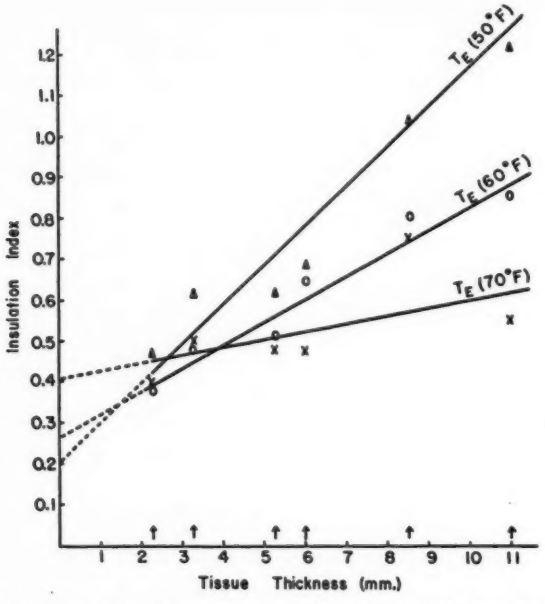

FIG. 1. Effect of one hour exposure, at certain environmental temperatures, on the insulation of tissues of varied thickness.

Considering the short exposures and also because of T_E used, it seems reasonable to make $T_R = 98.6°$ F. We have then calculated the thermal insulation indices after one hour of exposure at 70°, 60°, and 50° F. The results are reported in Table III and are illustrated in Fig. 1. The slopes in Fig. 1, which are statistically different from one another, give us the insulation of the fat layer, and the intercept, the insulation of the other tissues.

TABLE III

EFFECT OF SKIN AND SUBCUTANEOUS FAT THICKNESS ON THE INSULATION INDEX, AFTER ONE HOUR EXPOSURE AT DIFFERENT ENVIRONMENTAL TEMPERATURES

Subject	Thickness in mm.	Insulation index		
		70° F.	60° F.	50° F.
D.C.	2.25	0.40	0.39	0.47
G.S.	3.25	0.50	0.47	0.61
A.D.	5.25	0.48	0.51	0.62
J.L.	6.00	0.47	0.64	0.72
G.M.	8.50	0.76	0.80	1.04
F.R.	11.00	0.55	0.86	1.22

The noticeable shivering observed by the subjects at 20, 40, and 60 min. is reported in Table IV. It should be mentioned that whenever the subject was starting to shiver at 20 or 40 min., this shivering was more pronounced at 60 min.

TABLE IV

SHIVERING OBSERVED AT 20, 40, OR 60 MIN. WHEN THE ROOM TEMPERATURE WAS 70°, 60°, AND 50° F.

Thickness of epimuscular tissues (mm.)	Environmental temperatures		
	70° F.	60° F.	50° F.
2.25	—	40, 60	20, 40, 60
3.25	—	60	40, 60
5.25	—	—	40, 60
6.00	—	—	40, 60
8.50	—	—	60
11.00	—	—	60

The average skin temperature of the abdomen was lower than that of the chest; this finding may be explained by the difference in fat thickness observed between these two parts of the body. It was also noted that in thin subjects the temperature is somewhat higher in the front than in the back, whereas in fat subjects the temperature of the back is higher than that of the front. This observation may also be explained by the difference observed in the distribution of fat in thin and fat persons. Accordingly, it may be said that the variation in fat thickness explains not only the difference between the average skin temperatures of different persons, but also, to a certain extent, the regional variations of skin temperature encountered over the body. Finally, we observed that the temperature of the forehead, taken after 20, 40, and 60 min. at 70° F. is the same for all the subjects, i.e. about 93° F. Since the fat thickness on the forehead is approximately the same for all subjects, this result is in accordance with the general equation that we have found.

Discussion

None of the subjects shivered at 70° F. and the fat thickness, at this temperature, did not give the subjects any appreciable amount of insulation as evidenced in Fig. 1. These findings indicate that the nonfat tissues are probably sufficient to maintain the body in a thermal steady state, when the ambient temperature is 70° F., without an increased heat production. We hope to answer this question more affirmatively by making determinations of oxygen consumption.

At 60° F., however, subject D.C. (where I_T has not changed) would not be able to remain at a steady state if the metabolism is not increased. This is evidenced by the shivering observed in this subject (Table IV). But in the subjects who have more fat, this would not be the case; I_F changes and allows a steady state without an increase in heat production.

At 50° F. all the subjects shivered, but the onset was slower in fat persons. Consequently, it seems that even in the fat subjects, the insulation was not sufficient to maintain a steady state without a simultaneous increased heat production.

Finally, using the equation $[1.21\ (I_F + I_B)]/I_A$ = thermal insulation index, we find, making $I_A = 0.8$ Clo Units (3) (since exposures were made in a still air room), the following values for I_F, I_B and I $(I_F + I_B + I_A)$ when the thickness of the fat layer is one inch.

T_E (° F.)	I_F	I_B	I
70	0.41	0.41	1.62
60	1.18	0.26	2.24
50	1.96	0.21	2.97

Conclusions

(1) As expected, the fall of skin temperature is larger at the beginning than at the end of the exposure and the rate of fall is faster at 70°F. than at 60° or 50° F.

(2) Results obtained show that the insulation of the fat layer increases as the environmental temperature is lowered.

(3) The variations in fat thickness explain not only the differences in average skin temperature of different persons, but also, to a certain extent, the regional variations of skin temperatures observed over the body.

Acknowledgment

The author gratefully acknowledges the co-operation of the test subjects and is indebted to Dr. G. F. M. Smith and Miss M. Fleming, who performed the statistical analyses.

References

1. Burton, A. C. J. Nutrition, 7 (No. 5) : 481. 1934.
2. Edwards, D. A. W. Clin. Sci. 9 : 259. 1950.
3. Gagge, A. P., Burton, A. C., and Bazett, H. C. Science, 94 : 428. 1941.
4. Glaser, E. M. J. Physiol. 109 : 366. 1949.
5. Hardy, J. D., Milhorat, A. T., and DuBois, E. F. Temperature, its measurement and control in science and industry. Reinhold Publishing Corporation, New York, N.Y. 1941. p. 529.
6. Hatfield, H. S. and Pugh, L. G. C. Nature, 168 : 918. 1951.

THE LIPID AND WATER CONTENT OF CARCASS, SKELETAL MUSCLE, AND TESTICLE IN THE HOST COMPONENT OF THE ALBINO RAT – WALKER CARCINOMA 256 DUAL ORGANISM AT PROGRESSIVE STAGES OF TUMOR GROWTH[1]

By Eldon M. Boyd, Carl E. Boyd, J. Gilbert Hill, and Ely Ravinsky

Abstract

The objective of this investigation was to find at what stage in the growth of Walker carcinoma 256 appear the shifts in water and lipid levels of host carcass, skeletal muscle, and testicle, noted at or near death of the dual organism by Boyd, Connell, and McEwen (1952). Lipid and water estimations were made upon these tissues, at intervals of one, two, and three weeks of tumor growth, in 35 tumor-bearing and 34 littermate control albino rats. In host carcass, the decline in concentration, per 100 gm. dry weight, of total lipid, neutral fat, and total fatty acids appeared after two weeks of tumor growth, while at or about the same time a rise occurred in the levels of water, total cholesterol, free cholesterol, and phospholipid. In hind limb skeletal muscle of the host, the levels of total lipid, neutral fat, and total fatty acids were lowered, while those of water were elevated, after two and three weeks of tumor growth. In host testicle, the levels of water and lipids were essentially similar to those of the controls. The rise in concentration of water, phospholipid, total cholesterol, and free cholesterol of the host varied, in general, with increase in the T/RC coefficient. Maximal low levels of host total lipid, neutral fat, and total fatty acids were reached at T/RC coefficient values of 20 to 30. Maintenance of total body weight (tumor plus host) was due mainly to accumulation of water in both components. The host component lost dry weight, total lipid, neutral fat, and total fatty acids more rapidly than these accumulated in the tumor in total amount. The smaller loss of total amounts of water, phospholipid, total cholesterol, and free cholesterol in the host was offset by an approximately equal accumulation of these substances in the tumor.

Introduction

The investigation to be described below was a continuation of studies which have been in progress in this department at Queen's University since 1948, on the interrelationships of lipid and water metabolism in the albino rat – Walker carcinoma 256 dual organism (1). The tumor component of this dual organism was found by Boyd and McEwen (2) to maintain, throughout its growth in the host albino rat, a relatively high level of phospholipid, free cholesterol, ester cholesterol, and water and a relatively low level of neutral fat. The carcass of littermate albino rats without tumors, of "normal controls," has the opposite lipid and water levels (1). Skeletal muscles of the thigh and hind leg have what might be described as less opposite or intermediary lipid and water levels (1). The lipid and water concentrations in testicle are similar to those of the tumor component (1).

In the carcass, hind limb skeletal muscles, and testicles of the host component of the tumor–host organism, Boyd *et al.* (1) found a shift in the levels of lipids and water at or near death of the dual organism. The direction of the shift was toward the level of water and of the corresponding lipids in the tumor component in almost all instances. These changes suggest the production by the Walker tumor of a hydrolipotropic factor whose effect upon the

[1] *Manuscript received February 5, 1954.*
A contribution from the Department of Pharmacology, Queen's University, Kingston, Ontario.

tissues of the host is to make their water and lipid concentrations become more like those in the tumor component. Boyd *et al.* (3) reported that this hydrolipotropic shift was pronounced in skin and gut, much less evident in liver, lung, and heart and apparently absent in brain.

Since the hydrolipotropic shift in host carcass is of primary consideration in this study of tumor–host relationships, it was decided to investigate this shift further, before extending the research to organs and tissues remaining to be analyzed. Hence a study has been made of the water and lipid content of host carcass at progressive stages of tumor growth. Host hind limb skeletal muscle and host testicle were included as representing tissues affected to an intermediary and slight extent by the hydrolipotropic shift.

Method

A description of the transplantable Walker carcinoma 256, as maintained in the laboratories and animal quarters of the Department of Pharmacology at Queen's University since 1947, has been given in previous reports (1, 2, 3). That the tumor has attained increased virulence, in the form of increased capacity to grow, is evident by a comparison of weekly tumor weights as given in Table I herein and in Table I of the report of Boyd and McEwen in 1952 (2).

TABLE I

THE WEIGHT PARTITION OF THE ALBINO RAT – WALKER CARCINOMA 256 DUAL ORGANISM AT PROGRESSIVE INTERVALS OF TUMOR GROWTH, AND OF LITTERMATE CONTROLS WITHOUT TUMORS

Weeks of tumor growth	Number of animals	Calculation	Inoculation weight (gm.)	Final weight (gm.)	Tumor weight (gm.)	Residual carcass weight (gm.)	*T/RC* coefficient
Tumor present							
1	12	Mean	79	114	1.7	112.3	1.4 •
		Standard deviation	31	27	1.4	25.7	1.2
2	12	Mean	87	183	42	141	30
		Standard deviation	20	35	23	32	21
3	11	Mean	99	210	58	152	38
		Standard deviation	35	58	24	41	23
Tumor absent							
1*	12	Mean	80	123			
		Standard deviation	26	22			
2*	11	Mean	87	173			
		Standard deviation	19	28			
3*	11	Mean	103	204			
		Standard deviation	37	39			

* *Corresponding growth interval in littermate controls without tumors.*

Tumor-bearing male albino rats and littermate control males without tumors were sacrificed at the end of one, two, and three weeks of tumor growth in the numbers indicated in Table I, 69 animals in all being used. Data upon body weights at the time of inoculation and at the time of sacrifice and, in the tumor-bearing animals, data upon weights of tumors and residual carcasses have been summarized and presented in Table I. The animals were fed Purina fox chow checkers and water ad libitum. Doors to rooms which housed the albino rats were kept locked and no foreign albino rats were introduced to the colony during this investigation.

Aliquots of hind limb skeletal muscles, testicle, and minced residual carcass were taken for lipid and water analysis after the techniques described by Boyd *et al.* (1). The results obtained were summarized statistically, after the criteria of Bradford Hill (8).

Results

Data obtained upon carcass are presented in Table II. From these figures, differences between means in the tumor-bearing albino rats and in the littermate controls at the 99% level of significance (8) or better will be noted and trends indicated. Water concentration in host carcass rose above the level in the controls after two weeks of tumor growth and remained above at three weeks. Total lipid, neutral fat, and total fatty acid levels, per 100 gm. dry weight of carcass, had significantly declined in the host rat after two weeks of tumor growth and were lower still at three weeks. On the other hand, the concentration of total cholesterol remained relatively constant, with an over-all mean increase of 17%. At two and three weeks of tumor growth, there was a mean increase of 19% in carcass free cholesterol levels, with no significant trends in the low and variable concentration of ester cholesterol. The concentration of phospholipid was elevated even after one week of tumor growth and the average increase over the three weeks was 24%.

The results derived from analysis of skeletal muscle are presented in Table III and again differences of 99 per cent statistical significance or better will be noted. The concentrations of total lipid, neutral fat, and total fatty acids in skeletal muscle of the host fell after two weeks of tumor growth and remained lower than values in the littermate controls at three weeks. The P value of increases in the mean levels of the cholesterol fractions and phospholipid shown in Table III was less than 0.01. Water content of this tissue increased after two and three weeks of tumor growth.

From Table IV it may be observed that, in the testicle, there was an increase in the three cholesterol levels at two weeks and in the phospholipid level at three weeks with no other differences between concentration of water and lipids in the tumor-bearing and littermate control albino rats.

These findings may be compared with those of Boyd *et al.* (1) who analyzed the same tissues after four weeks of tumor growth. The decrease in total lipid, neutral fat, and total fatty acids of the host carcass, and the increase in water, phospholipid, and free cholesterol previously found antemortally in the

same tissue, have been shown to become evident at the two week interval, or mid-point, of tumor growth. The same findings in hind limb skeletal muscle of host albino rats bearing four-week-old tumors have been shown to be present also at the mid-point of tumor–host growth, with the exception of the four week increase in free cholesterol and phospholipid. The water and lipid content of testicle remain relatively constant throughout growth of the tumor in the host.

TABLE II

THE WATER AND LIPID CONTENT OF CARCASS IN THE HOST COMPONENT OF THE ALBINO RAT – WALKER CARCINOMA 256 DUAL ORGANISM, AND IN LITTERMATE CONTROLS WITHOUT TUMORS, DURING PROGRESSIVE STAGES OF TUMOR GROWTH

(Values for water are expressed as gm. per 100 gm. wet weight, and for lipids as gm. per 100 gm. dry weight, of carcass)

Analysis	Tumor	Calculation	Weeks of tumor growth					
			1		2		3	
			No. of animals	Value	No. of animals	Value	No. of animals	Value
Water	Present	Mean	6	70.2	10	72.9	10	71.7
		Standard deviation		2.1		2.9		2.5
	Absent	Mean	6	71.2	8	68.1	10	68.0
		Standard deviation		0.7		1.9		2.4
Total lipid	Present	Mean	6	10.09	10	6.46	9	5.82
		Standard deviation		3.01		2.25		2.56
	Absent	Mean	6	10.43	7	13.16	10	16.75
		Standard deviation		2.42		2.65		4.50
Neutral fat	Present	Mean	6	5.97	10	1.76	9	2.43
		Standard deviation		2.86		1.64		3.01
	Absent	Mean	6	7.17	7	9.48	10	13.10
		Standard deviation		2.07		2.14		3.81
Total fatty acids	Present	Mean	6	7.84	10	4.30	10	3.81
		Standard deviation		2.63		1.97		2.52
	Absent	Mean	6	8.59	8	10.89	10	14.60
		Standard deviation		2.17		2.45		4.23
Total cholesterol	Present	Mean	6	0.71	10	0.77	10	0.56
		Standard deviation		0.13		0.12		0.13
	Absent	Mean	6	0.63	8	0.49	10	0.63
		Standard deviation		0.06		0.04		0.36
Ester cholesterol	Present	Mean	6	0.19	8	0.18	10	0.12
		Standard deviation		0.19		0.11		0.06
	Absent	Mean	6	0.11	8	0.08	10	0.19
		Standard deviation		0.04		0.05		0.36
Free cholesterol	Present	Mean	6	0.52	8	0.54	10	0.51
		Standard deviation		0.09		0.11		0.10
	Absent	Mean	6	0.52	8	0.41	10	0.44
		Standard deviation		0.09		0.05		0.07
Phospholipid	Present	Mean	6	3.31	10	3.95	9	3.43
		Standard deviation		0.36		1.54		0.79
	Absent	Mean	6	2.56	7	3.15	10	2.90
		Standard deviation		0.60		0.93		0.67

TABLE III

THE WATER AND LIPID CONTENT OF SKELETAL MUSCLE IN THE HOST COMPONENT OF THE ALBINO RAT – WALKER CARCINOMA 256 DUAL ORGANISM, AND IN LITTERMATE CONTROLS WITHOUT TUMORS, DURING PROGRESSIVE STAGES OF TUMOR GROWTH

(Values for water are expressed as gm. per 100 gm. wet weight, and for lipids as gm. per 100 gm. dry weight, of skeletal muscle)

Analysis	Tumor	Calculation	Weeks of tumor growth					
			1		2		3	
			No. of animals	Value	No. of animals	Value	No. of animals	Value
Water	Present	Mean	12	76.8	12	79.5	11	80.1
		Standard deviation		1.1		2.1		3.0
	Absent	Mean	12	77.2	11	76.0	11	75.4
		Standard deviation		1.6		0.8		1.1
Total lipid	Present	Mean	12	10.39	12	6.48	8	7.12
		Standard deviation		3.00		1.70		2.24
	Absent	Mean	11	11.42	11	10.16	11	9.98
		Standard deviation		3.53		2.70		2.92
Neutral fat	Present	Mean	12	5.22	10	1.48	8	2.41
		Standard deviation		2.32		1.52		2.14
	Absent	Mean	11	6.46	11	4.73	11	5.72
		Standard deviation		1.73		3.32		2.83
Total fatty acids	Present	Mean	12	8.13	12	4.37	11	4.43
		Standard deviation		2.66		1.70		2.35
	Absent	Mean	12	9.09	11	7.87	11	8.02
		Standard deviation		2.24		2.52		2.91
Total cholesterol	Present	Mean	12	0.38	12	0.47	11	0.44
		Standard deviation		0.10		0.17		0.22
	Absent	Mean	12	0.40	11	0.41	11	0.36
		Standard deviation		0.08		0.27		0.13
Ester cholesterol	Present	Mean	12	0.04	10	0.04	10	0.10
		Standard deviation		0.11		0.04		0.09
	Absent	Mean	12	0.04	11	0.10	11	0.02
		Standard deviation		0.04		0.23		0.03
Free cholesterol	Present	Mean	12	0.35	10	0.45	10	0.37
		Standard deviation		0.05		0.19		0.10
	Absent	Mean	12	0.36	11	0.31	11	0.36
		Standard deviation		0.08		0.05		0.20
Phospholipd	Present	Mean	12	4.76	12	4.97	10	4.05
		Standard deviation		1.38		1.26		0.71
	Absent	Mean	11	4.51	11	4.96	11	3.94
		Standard deviation		1.92		1.05		0.25

TABLE IV

THE WATER AND LIPID CONTENT OF TESTICLE IN THE HOST COMPONENT OF THE ALBINO RAT – WALKER CARCINOMA 256 DUAL ORGANISM, AND IN LITTERMATE CONTROLS WITHOUT TUMORS, DURING PROGRESSIVE STAGES OF TUMOR GROWTH

(Values for water are expressed as gm. per 100 gm. wet weight, and for lipids as gm. per 100 gm. dry weight, of testicle)

Analysis	Tumor	Calculation	Weeks of tumor growth					
			1		2		3	
			No. of animals	Value	No. of animals	Value	No. of animals	Value
Water	Present	Mean	8	86.5	9	87.2	9	87.1
		Standard deviation		1.1		0.3		1.7
	Absent	Mean	10	87.0	11	87.3	11	86.7
		Standard deviation		0.2		0.4		0.3
Total lipid	Present	Mean	12	12.50	11	13.60	11	13.16
		Standard deviation		4.00		1.72		2.14
	Absent	Mean	12	13.09	11	12.72	11	12.20
		Standard deviation		2.97		1.00		2.31
Neutral fat	Present	Mean	12	0.98	11	1.39	11	0.50
		Standard deviation		1.32		1.42		0.72
	Absent	Mean	12	0.95	11	0.78	11	1.74
		Standard deviation		1.22		0.84		2.34
Total fatty acids	Present	Mean	12	7.35	11	8.46	11	7.76
		Standard deviation		1.78		1.12		1.61
	Absent	Mean	12	7.23	11	7.92	11	7.67
		Standard deviation		1.35		0.61		1.95
Total cholesterol	Present	Mean	12	1.17	12	1.44	11	1.13
		Standard deviation		0.28		0.24		0.35
	Absent	Mean	12	1.25	11	1.18	11	1.13
		Standard deviation		0.33		0.11		0.21
Ester cholesterol	Present	Mean	12	0.11	9	0.26	11	0.01
		Standard deviation		0.16		0.21		0.29
	Absent	Mean	12	0.08	11	0.11	11	0.00
		Standard deviation		0.14		0.08		0.17
Free cholesterol	Present	Mean	12	1.06	9	1.18	11	1.12
		Standard deviation		0.32		0.13		0.14
	Absent	Mean	12	1.17	11	1.07	11	1.16
		Standard deviation		0.18		0.07		0.40
Phospholipid	Present	Mean	12	9.76	12	10.75	11	11.82
		Standard deviation		3.52		1.67		1.95
	Absent	Mean	12	10.87	11	10.68	11	9.62
		Standard deviation		3.44		0.94		1.64

The *T/RC* Coefficient

When the data exhibiting a 99% statistically significant difference from the controls were plotted against an index of tumor growth, further interrelationships of water and lipid metabolism in the tumor–host dual organism became evident. Scattergrams were prepared by plotting water and lipid levels in the host against the *T/RC* coefficient. The latter coefficient was calculated as follows: the wet weight of the tumor was divided by the wet weight of the residual carcass, and the resulting fraction was multiplied by 100 to give the

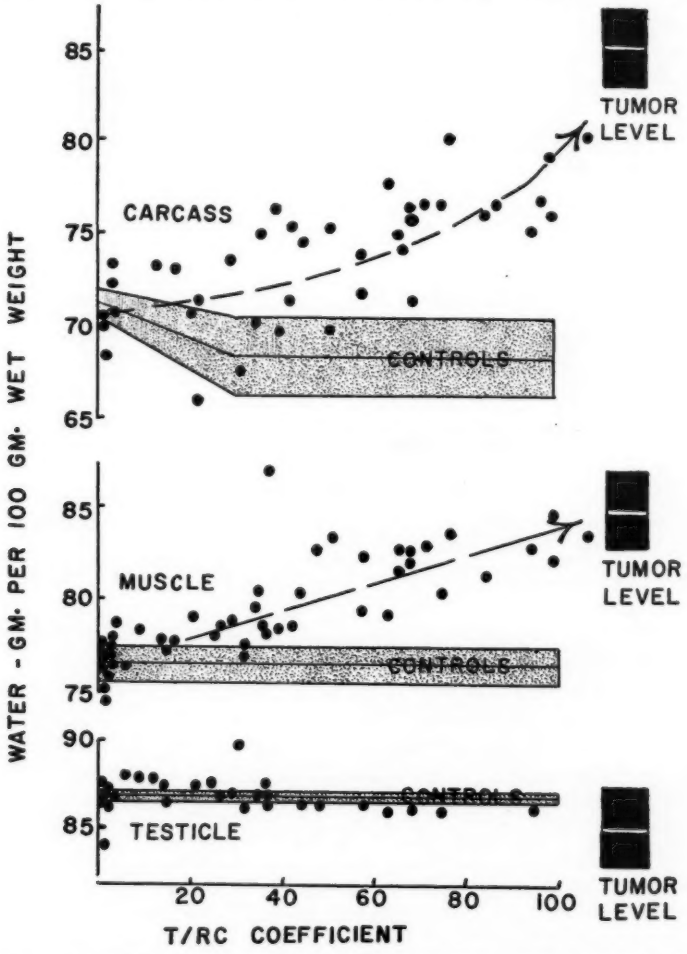

FIG. 1. The relation of the water concentration of host carcass, hind limb skeletal muscle, and testicle to increasing values of the *T/RC* coefficient. (The mean ± standard deviation of corresponding water concentrations in littermate albino rats without tumors is shown as stippled areas and in the tumor as solid areas.)

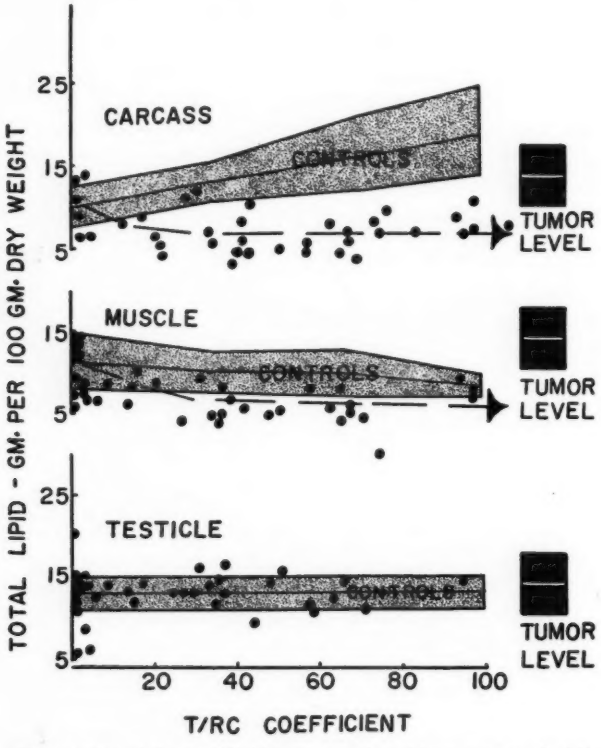

FIG. 2. The relation of the total lipid concentration of host carcass, hind limb skeletal muscle, and testicle to increasing values of the T/RC coefficient. (The mean ± standard deviation of corresponding total lipid concentrations in littermate albino rats without tumors is shown as stippled areas and in the tumor as solid areas.)

T/RC coefficient. To present a picture of the complete life history of the tumor–host organism, data used in preparing a previous report (1) were analyzed again and used as indicated. Changes in the host component of the dual organism were compared to the values of littermate controls over the same period of growth by plotting the means and standard deviations of the controls as shadowgrams. For comparative purposes, there was included the range of the mean ± standard deviation of corresponding analyses of the terminal tumor, as calculated after the data of Boyd and McEwen (2) and of Boyd *et al.* (3).

Such a diagram of progressive changes in water concentration is presented in Fig. 1, in which are plotted some 40 to 50 analyses in each of host and control carcass, hind limb skeletal muscle and testicle and 24 analyses of tumor. The carcass of albino rats without tumors contained a much lower water concentration than did tumor. As the tumor grew, the water concentration of the host increased rapidly toward levels in the tumor. The muscles

of the hind limb of albino rats without tumors contained a water concentration moderately below that of tumor. As the tumor grew, the water level in these muscles rose. The water level in testicle of tumorless rats was within the upper range of water levels in the tumor and was not appreciably affected by tumor growth.

A corresponding representation of alterations in tumor–host total lipid levels is given in Fig. 2. As may be seen, the changes in carcass and skeletal muscle progressed in the opposite direction to those plotted in Fig. 1, while again there were no significant alterations in the tumorlike levels of testicular total lipid. The maximum decrease in total lipid levels of host carcass and hind limb skeletal muscle occurred at T/RC coefficients of about 20 to 30 and no further trend to lower levels was evident with increasing values of the T/RC coefficient.

The relation of concentrations of neutral fat and total fatty acids in host carcass to increasing values of the T/RC coefficient is shown in Fig. 3. When the coefficient rose to around 20, the levels of both of these lipids fell to minimal low values, which were maintained throughout the balance of tumor growth.

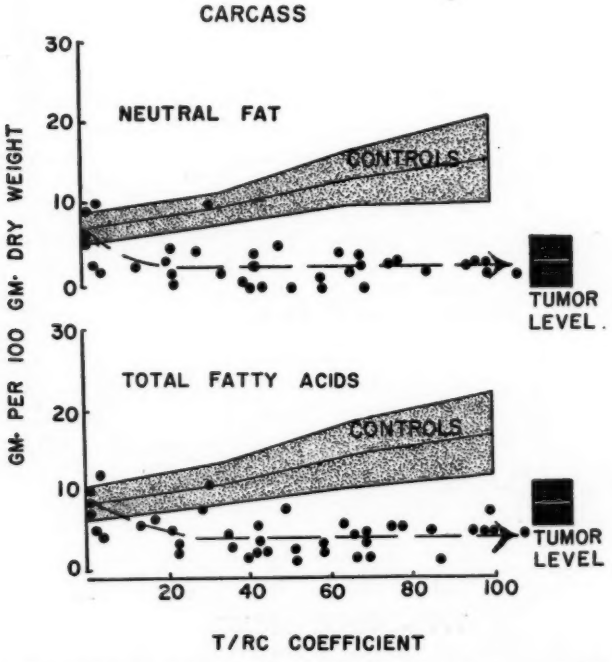

FIG. 3. The relation of the neutral fat and total fatty acid concentrations of host carcass to increasing values of the T/RC coefficient. (The mean ± standard deviation of corresponding neutral fat and total fatty acid concentrations in littermate albino rats without tumors is shown as stippled areas and in the tumor as solid areas.)

The concentrations of phospholipid, total cholesterol, and free cholesterol in the host carcass increased slowly as the *T/RC* coefficient rose, as shown in Fig. 4. As is evident in Figs. 3 and 4, changes in the concentration of neutral fat, total fatty acids, phospholipid, total cholesterol, and free cholesterol in the host carcass were toward the levels of the corresponding lipids in the tumor.

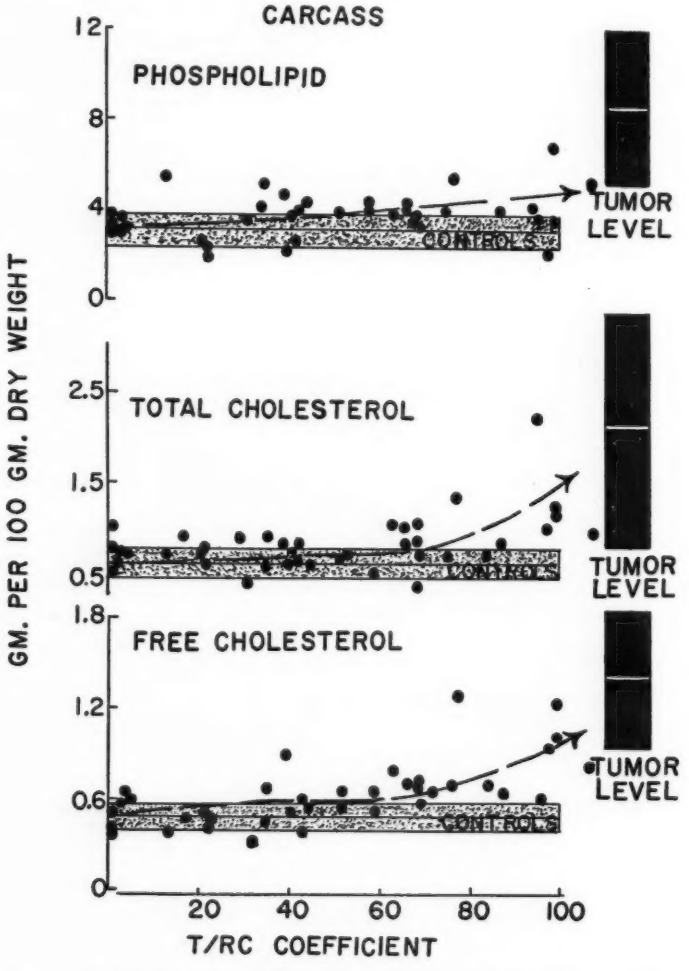

FIG. 4. The relation of the phospholipid, total cholesterol, and free cholesterol concentrations of host carcass to increasing values of the *T/RC* coefficient. (The mean ± standard deviation of corresponding phospholipid, total cholesterol, and free cholesterol concentrations in littermate albino rats without tumors is shown as stippled areas and in the tumor as solid areas.)

Discussion

From the evidence presented above, it is apparent that both water concentration and dry weight composition of host carcass vary with increasing growth of Walker carcinoma 256. Thus, when concentrations are expressed as gm. per 100 gm. dry weight of carcass, two variables are involved. Concentrations expressed as gm. per 100 gm. of nonlipid dry weight of carcass would be indicated from a consideration of the evidence presented in this report alone. However, nonlipid dry weight of carcass in rats bearing Walker carcinoma 256 is also a variable value. Mider *et al.* (10), who have reviewed earlier relative literature, found a decline in the concentration of nitrogen in host carcass, with increasing growth of Walker carcinoma 256, and this was later confirmed by Sherman *et al.* (11). Le Page *et al.* (9) used glycine-2-C^{14} to prove that there was decreased synthesis of protein and nucleic acids in the bodies of rats bearing Flexner-Jobling carcinoma. These quoted findings indicate that a loss of protein occurs in the dry weight fraction of host carcass, during tumor growth.

Therefore, the data described under "Results" above indicate that, *relative to dry weight*, there is a decrease in total lipid, neutral fat, and total fatty acids in the host carcass, and an increase in total cholesterol, free cholesterol, and phospholipid, with increasing growth of Walker carcinoma 256. As may be calculated from data given in Tables I and II above and supplied by Boyd *et al.* (1), there is a progressive decline in the growth rate of the host, calculated as dry weight and compared with corresponding values in littermate controls without tumors. The total amount of lipids, water, and dry weight lost by the host, per kgm. of tumor–host dual organism, was therefore calculated. This was done by calculating the mean total amount of each constituent in the host, subtracting this value from the corresponding value in littermate controls without tumors, and expressing the difference as gm. per kgm. body weight of tumor–host dual organism. The results are presented in Table V. The accumulation of lipids in the tumor component was similarly calculated and presented in Table V of the paper by Boyd and McEwen (2).

TABLE V

THE LOSS OF DRY WEIGHT, WATER, AND LIPIDS IN THE HOST COMPONENT OF THE ALBINO RAT – WALKER CARCINOMA 256 DUAL ORGANISM AT PROGRESSIVE STAGES OF TUMOR GROWTH

(The results are expressed as gm. per kgm. of tumor–host organism)

Analysis	Weeks of tumor growth			
	1	2	3	4
Water	77.8	80.9	143	241
Dry weight	17.5	92.8	95.8	162
Total lipid	2.72	26.1	38.5	46.3
Neutral fat	4.82	25.2	34.5	41.7
Total fatty acids	3.68	23.8	36.2	42.2
Total cholesterol	+ 0.17	+ 0.16	0.76	0.60
Ester cholesterol	+ 0.17	+ 0.16	0.33	0.13
Free cholesterol	0.00	0.11	0.29	0.47
Phospholipid	+ 1.84	1.20	1.71	4.20

A balance sheet demonstrating shifts in total amounts of lipids, dry weight, and water in the tumor component and host component was drawn up from data obtained in this study and investigations previously reported from this department at Queen's University (1, 2, 3). This balance sheet is presented in Fig. 5. The balance sheet demonstrates that the maintenance of tumor–host weight was due to accumulation of water in both tumor and host. Dry weight, total lipid, neutral fat, and total fatty acids were progressively lost by the host. The gain of these elements in the tumor did not compensate for their loss in the host. On the other hand, the amount of phospholipid, total cholesterol, and free cholesterol lost by the host, with increasing tumor growth, was much less and was compensated by accumulation of these lipids in the tumor component.

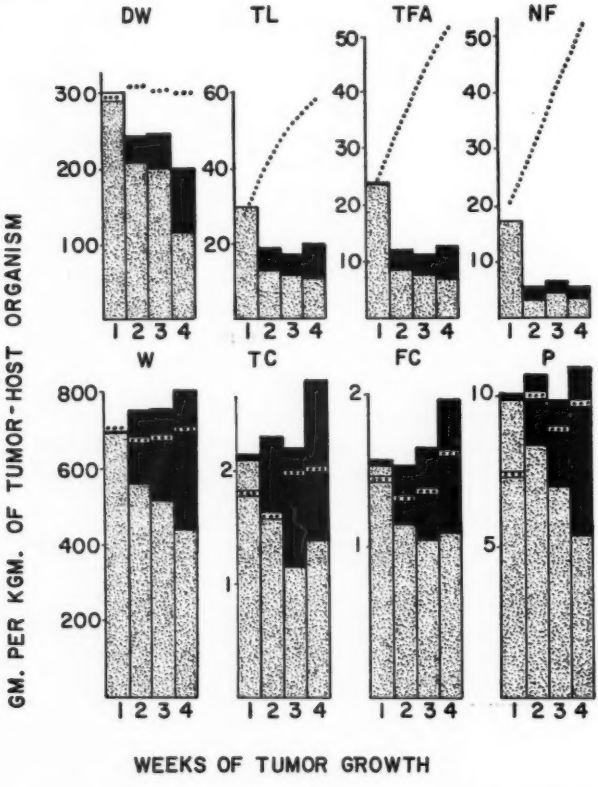

FIG. 5. The total amount of dry weight, water, and lipids in the host (stippled areas), tumor (solid areas), and littermate albino rats without tumors (dotted lines) at progressive stages of tumor growth. (Legend: DW–dry weight; TL–total lipid; TFA–total fatty acids; NF–neutral fat; W–water; TC–total cholesterol; FC–free cholesterol; P–phospholipid.)

These several calculations indicate that Walker carcinoma 256 has an effect upon host water, total cholesterol, free cholesterol, and phospholipid different from that upon host total lipid, neutral fat, total fatty acids, and dry weight. As shown in Fig. 5, relatively large amounts of water, total cholesterol, free cholesterol, and phospholipid accumulate in the tumor and begin to do so even after two weeks of tumor growth. Yasuda (14) demonstrated in 1931, by using dietary fat of high and low iodine number, that phospholipid of experimental tumors is built from fatty acids ingested by the host. More recently, Costello *et al.* (4) reported that P^{32} injected subcutaneously as sodium P^{32} phosphate, rapidly accumulates in the phospholipid fraction of methylcholanthrene-induced mouse carcinoma.

The general effects of tumors upon host carcass, tissues, and organs have been recently reviewed (6, 7). Earlier literature relevant to the lipid and water metabolism of the albino rat – Walker carcinoma 256 dual organism has been reviewed in previous reports from this department at Queen's University (1, 2, 3). More recently, Stewart and Begg (12, 13) have shown that force feeding of rats bearing this tumor inhibits, but does not completely prevent, loss of carcass weight, nitrogen, and fatty acids. The concept of tumor elaborating a factor which affects the metabolism of host tissues has been mentioned in earlier literature and more recently by Greenfield and Meister (5), who reported that alcoholic extracts of acetone-precipitated tumor tissue lowered the catalase activity of mouse liver.

Acknowledgments

The authors wish to acknowledge the assistance and cooperation of Marion A. Boyd, J. Coates, Jr., Elizabeth A. Hineman, H. D. McEwen, and Joan K. Miller. This investigation was aided by a grant from the National Cancer Institute of Canada.

References

1. Boyd, E. M., Connell, M. L., and McEwen, H. D. Can. J. Med. Sci. 30 : 471. 1952.
2. Boyd, E. M. and McEwen, H. D. Can. J. Med. Sci. 30 : 163. 1952.
3. Boyd, E. M., McEwen, H. D., and Shanas, M. N. Can. J. Med. Sci. 31 : 493. 1953.
4. Costello, C. J., Carruthers, C., Kamen, M. D., and Simoes, R. L. Cancer Research, 7 : 642. 1947.
5. Greenfield, R. E. and Meister, A. J. Natl. Cancer Inst. 11 : 997. 1951.
6. Greenstein, J. P. Biochemistry of cancer. Academic Press Inc., New York. 1947.
7. Greenstein, J. P. and Haddow, A. Advances in cancer research. Academic Press Inc., New York. 1953.
8. Hill, A. B. Principles of medical statistics. 2nd ed. The Lancet Limited, London. 1939.
9. Le Page, G. A., Potter, V. R., Busch, H., Heidelberger, C., and Hurlbert, R. B. Cancer Research, 12 : 153. 1952.
10. Mider, G. B., Tesluk, H., and Morton, J. J. Acta Union Internat. contre Cancer, 6 : 409. 1948.
11. Sherman, C. D., Morton, J. J., and Mider, G. B. Cancer Research, 10 : 374. 1950.
12. Stewart, A. G. and Begg, R. W. Cancer Research, 13 : 556. 1953.
13. Stewart, A. G. and Begg, R. W. Cancer Research, 13 : 568. 1953.
14. Yasuda, M. Proc. Soc. Exptl. Biol. Med. 28 : 1074. 1931.

THE EFFECT OF CORTISONE ACETATE ON THE PRODUCTION[1] OF CIRCULATING HEMOLYTIC ANTIBODIES IN THE MOUSE

By Shirley E. Newsom and Marvin Darrach

Abstract

Quantitative immunochemical methods have been used to show that in the mouse cortisone acetate suppresses the formation of circulating hemolytic antibodies to sheep erythrocytes. The amount of suppression varies with the dose of both cortisone acetate and antigen. Experimental conditions are defined whereby an almost complete inhibition of circulating antibody occurs. The method serves as a quantitative procedure for comparing the effect of different steroids and hormones on antibody production as well as a means of studying possible cortisone antagonists.

Introduction

Published experiments involving quantitative immunochemical techniques have shown that the administration of cortisone acetate interferes with the production of circulating antibodies to certain antigens in the rabbit and guinea pig (1, 3, 4, 5). However, considerable variation is reported in the extent to which the serum levels of antibodies are suppressed from average normal levels. Since a demonstration of the activity of cortisone acetate in this regard may depend, among other factors, on the amount and kind of antigen, the species of animal, and the dose of hormone, the degree of antibody suppression induced by cortisone acetate will undoubtedly vary under different experimental conditions. Nevertheless, the statistically significant data given by Germuth and Ottinger (5) established conclusively that in the rabbit, cortisone acetate can inhibit almost completely the rise of circulating antibodies to crystalline egg albumin.

During preliminary experiments in these laboratories concerning the action of cortisone and other steroids on antibody production, it was observed that cortisone acetate, in the mouse, caused a marked suppression of the normal rise of circulating hemolytic antibodies to sheep erythrocytes. The following investigation was therefore designed to study this reaction in more detail and to develop a quantitative method for comparing the activity of cortisone acetate with that of other steroids and hormones on hemolytic antibody production.

Experimental

Preparation of Antigen

Sheep erythrocytes.—Sheep blood was collected by aseptic methods into sterile Alsever's solution (6). A portion of the red cells was centrifuged, washed three times at room temperature, and brought to a 1.0% suspension in sterile saline to serve as antigen. The remainder of the citrated blood was

[1] *Manuscript received February 23, 1954.*
Contribution from Department of Biochemistry, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia.

stored in the refrigerator as a source of erythrocytes for hemolytic antibody determinations. Thus, in each experiment, cells from a fresh blood sample were used for preparing antigen and the same sample provided cells for analyzing the corresponding antisera.

Immunization Procedure and Preparation of Antisera

Animals in each experimental and control group, consisting of 10 or 20 male or female Swiss mice, were selected so that variations in individual weights did not exceed 2 gm. The average weights of the males in different experiments ranged from 21–23 gm. and of the females from 18–20 gm.

Antigen was given by the intraperitoneal route; the standard dose was 0.1 ml. of a 1.0% suspension of sheep erythrocytes in sterile saline. Six days later all mice were anesthetized by ether and bled by cardiac puncture. After the pooled samples clotted, the sera were removed, clarified by centrifugation, and stored in the freezer.

Hemolysin Titers

Hemolytic end points were determined by mixing 0.2 ml. of a 1 : 20 dilution of reconstituted guinea pig serum,* 0.1 ml. of a 5% suspension of sheep erythrocytes, and 0.1 ml. of physiological saline with 0.4 ml. of doubling dilutions of antisera. After the mixtures were incubated for 30 min. in a 37.5° C. water bath, the degree of hemolysis was recorded for each tube.

Quantitative Comparison of Hemolytic Antibody

Mayer and associates (2, 7) demonstrated that in a standardized hemolytic system containing crystalline bovine albumin, veronal bicarbonate buffer, Ca^{++}, Mg^{++}, sheep erythrocytes, immune rabbit serum, and excess complement, the lytic process follows a kinetic curve similar to the reaction of an enzyme system with hemolytic antibody functioning as a catalyst. Furthermore, it was shown that the maximum rate of hemolysis during the reaction is a linear function of the concentration of hemolytic antibody. These observations make it possible to compare quantitatively the concentrations of hemolysins in different antisera.

The equipment and methods used in the present study for determining the hemolytic velocity curves of mouse antisera were similar to those described by Mayer for rabbit antisera, differing only in the following details. Complement was prepared from lyophilized guinea pig serum by reconstituting the dried powder with distilled water in 30 ml. portions and absorbing it with washed sheep erythrocytes as described by Mayer (7); several portions were pooled, subdivided into daily requirements and stored in the freezer. A 30% solution of bovine serum albumin** was used in the buffer and proved to be as satisfactory as crystalline albumin. The 125 ml. Erlenmeyer reaction flasks were shaken in a Warburg apparatus.

* *Mayer and Miles Laboratories, Allentown, Pa.*
** *Armour and Company, Chicago, Ill.*

Preliminary hemolytic end point titrations served as a guide for selecting antisera dilutions in the reaction mixtures. When concentrations of antisera in the 5.0 ml. portion added to the reaction mixture corresponded to the highest dilutions showing complete hemolysis in the preliminary titrations, the velocity curves were usually within the desired range. Such curves resulted from reactions producing 50–90% hemolysis in 30–60 min.

At recorded time intervals, samples were withdrawn during the course of the reaction, diluted 1 part with 2 parts of chilled citrate buffer (7), and centrifuged in the cold; a control flask, without antisera, was sampled in a similar manner. After the supernatants reached room temperature their optical densities were read against distilled water blanks in a Beckman DU spectrophotometer at a wave length of 543 mμ. These readings were plotted as a velocity curve and the maximum hemolytic rate was given by the slope of the curve corresponding to the greatest optical density change per minute. This rate multiplied by one-fifth the reciprocal of the dilution of serum in the 5.0 ml. portion added to the other components of the 25.0 ml. reaction mixture gave a number representing the potential hemolytic capacity of 1.0 ml. of the original serum. Such a value, referred to Mayer's standard system, expresses the hemolyzing potential of an antiserum in terms of change in optical density units per minute per ml. (O.D./min./ml.).

As illustrated in Fig. 1 the method developed by Mayer for measuring hemolytic antibodies in rabbit antisera applies equally well for the quantitative comparison of hemolysins in mouse antisera. The velocity curves shown represent different dilutions of a hemolytic mouse antiserum and the maximum

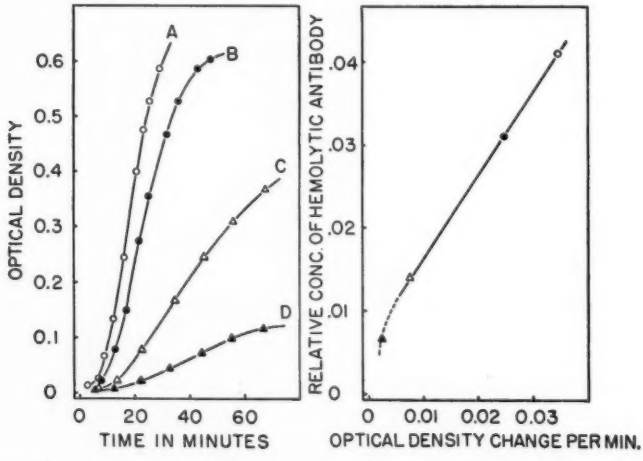

FIG. 1. Hemolytic velocity curves given by different dilutions of a mouse antiserum. A—1 : 24 dilution in the 5 ml. portion added to the 25 ml. reaction mixture. B—1 : 32 dilution. C—1 : 72 dilution. D—1 : 144 dilution. The second part of the diagram shows the linear relationship between antibody concentrations and the maximum rates of hemolysis.

reaction rate corresponding to each dilution, expressed as O.D. change per minute, is a linear function of the concentration of hemolytic antibody providing the rate is over 0.01 O.D./min. Such rates were always attained if at least 50% hemolysis took place after 60 min. incubation.

Effect of Erythrocytes from Different Sheep on the Maximum Rate of Hemolysis

Blood samples were taken from three sheep and suspensions of the erythrocytes were standardized in the usual manner (7). Duplicate velocity curves were determined for each red cell preparation in reaction mixtures containing the same concentration of a hemolytic mouse antiserum. The data in Table I show that reaction rates were not constant with different cell suspensions and support Mayer's statement that variations in lytic susceptibility occur among cells from different sheep (6). These results emphasize the importance of using the same red cell suspension throughout any given experiment.

TABLE I

VARIATIONS IN LYTIC SUSCEPTIBILITY OF ERYTHROCYTES FROM DIFFERENT SHEEP TO MOUSE HEMOLYTIC ANTIBODY

Sheep	Maximum rate of hemolysis* (O.D./min.)
U.B.C. – 3	.0153 .0158
U.B.C. – 5	.0265 .0241
U.B.C. – 6	.0154 .0167

* *Determined with pooled mouse hemolytic antisera No. 350–58.*

Time of Maximum Circulating Hemolytic Antibody Response in Mice After a Single Intraperitoneal Injection of Sheep Erythrocytes

Three groups of 20 male and three groups of 20 female mice were injected intraperitoneally with the standard dose of sheep erythrocytes. After three, six, and nine days a group of males and a group of females were bled and the pooled sera from each were frozen. At the end of the experiment, hemolytic rates were determined on aliquots of each pool of sera. The data in Fig. 2 show that maximum antibody response, at the time intervals tested, occurred six days after the injection of antigen.

Effect of Different Levels of Cortisone Acetate on Production of Circulating Hemolytic Antibodies in the Mouse

The following experiment was designed to determine the minimum dose of cortisone acetate required to produce a maximum suppression of hemolytic antibody in response to a constant level of antigen.

Forty male and 40 female mice were divided into groups of 10. On day 1 and day 4 three groups of each sex received subcutaneous injections of 0.1 mgm., 0.5 mgm., and 2.5 mgm. of cortisone acetate* respectively; the control groups received no hormone. All animals were given intraperitoneal injections

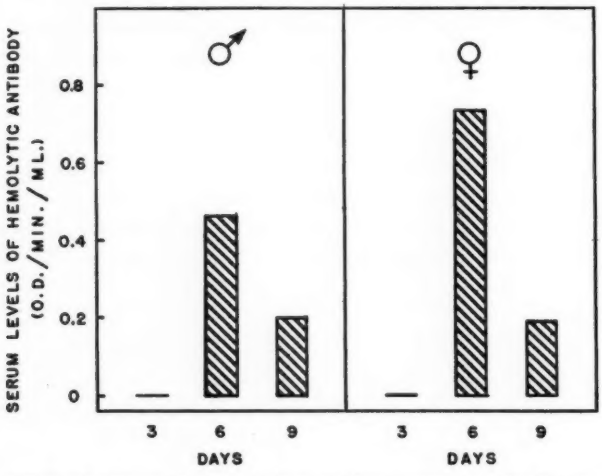

FIG. 2. Effect of time on the hemolytic antibody response to a single intraperitoneal injection of the standard amount of antigen. Each bar represents the hemolytic antibody level of sera pooled from 20 mice.

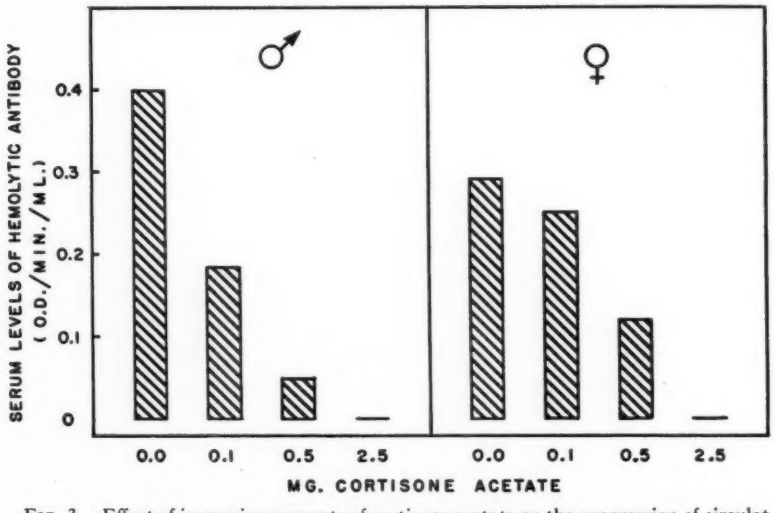

FIG. 3. Effect of increasing amounts of cortisone acetate on the suppression of circulating hemolytic antibody levels. The indicated doses were administered to each mouse on day 1 and 4. Sera were pooled from groups of 10 mice.

* *Merck Cortone.*

of the standard antigen dilution on day 2 and were bled six days later by cardiac puncture. Quantitative hemolytic antibody determinations were made on samples of each pool of sera. The results are given in Fig. 3. Whereas the 2.5 mgm. dose almost completely inhibited circulating hemolytic antibody, only partial suppression resulted from the 0.5 mgm. and 0.1 mgm. doses. In other experiments where amounts between 0.5 mgm. and 2.5 mgm. were tested, 2.0 mgm. did not give complete suppression in the female groups. Furthermore, it was found that the second injection of hormone at the 2.5 mgm. level on day 4 was unnecessary since the rise of antibody was checked by the first injection; the second dose was omitted thereafter. As shown in Table II, the results of several experiments have consistently demonstrated that a single subcutaneous injection of 2.5 mgm. of cortisone acetate given a day before the antigen, results in an almost complete inhibition of circulating hemolytic antibodies in both male and female Swiss mice. The data in Table III demonstrate that cortisone acetate similarly inhibits antibody production when the standard dose of antigen is administered by the intravenous route.

TABLE II

EFFECT OF A SINGLE 2.5 MGM. SUBCUTANEOUS INJECTION OF CORTISONE ACETATE ON CIRCULATING HEMOLYTIC ANTIBODY LEVELS IN MALE AND FEMALE SWISS MICE

Experiment*	Control animals			Cortisone acetate treated animals		
	No. in group	Sex	Hemolytic antibody level of pooled sera (O.D./min./ml.)	No. in group	Sex	Hemolytic antibody level of pooled sera (O.D./min./ml.)
1	10	♂	0.394	10	♂	0.007
	10	♀	0.594	10	♀	0.012
2	10	♂	0.398	10	♂	0.002
	20	♀	0.298	10	♀	0.003
3	10	♀	0.402	10	♂	0.009
				10	♂	0.002
4	10	♂	0.398	10	♂	0.019
	20	♀	0.134	10	♀	0.002
5	20	♂	0.377	10	♂	0.000
	20	♀	0.434	10	♀	0.009
6	20	♂	0.142	10	♂	0.002
	20	♀	0.415	10	♀	0.010
7	20	♂	0.178	10	♂	0.010
	20	♀	0.224	10	♀	0.003
8	10	♂	0.162	10	♂	0.000
	20	♀	0.591	10	♀	0.003
9	20	♀	0.154	10	♀	0.000

* *Each experiment was conducted with a different batch of sheep erythrocytes.*

TABLE III

EFFECT OF CORTISONE ACETATE* ON CIRCULATING HEMOLYTIC ANTIBODY FOLLOWING A SINGLE INTRAVENOUS INJECTION OF ANTIGEN

Control animals			Cortisone acetate treated animals		
No. in group	Sex	Hemolytic antibody level of pooled sera (O.D./min/ml.)	No. in group	Sex	Hemolytic antibody level of pooled sera (O.D./min./ml.)
9	♂	0.181	13	♂	0.002
8	♀	0.403	10	♀	0.000

** In this experiment 2.5 mgm. of cortisone acetate was given subcutaneously on both day 1 and day 4.*

Effect of Increasing Levels of Antigen on the Production of Circulating Hemolytic Antibodies in the Normal and Cortisone Acetate Treated Mouse

The following experiment was designed to study the effect on antibody production of increasing the amount of antigen while keeping the level of cortisone acetate constant.

Cortisone acetate (2.5 mgm.) was administered subcutaneously to each animal in four groups of 10 female mice. Four groups of 10 female mice served as controls. The following day different amounts of antigen were given intraperitoneally to each of the four cortisone acetate treated groups and the corresponding controls as indicated in Fig. 4. Each animal was bled by cardiac puncture six days later and the sera from each group were pooled and tested as before. As usual, 2.5 mgm. of cortisone acetate inhibited the

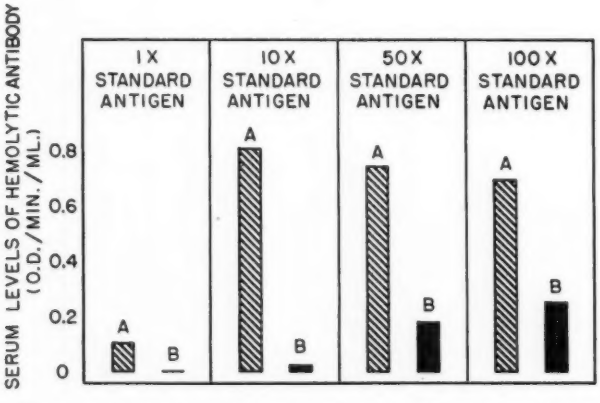

FIG. 4. Effect on circulating hemolytic antibody levels of increasing amounts of antigen in normal mice and mice receiving a constant amount of cortisone acetate. A—groups receiving antigen only. B—groups receiving antigen and 2.5 mgm. cortisone acetate per mouse.

appearance of hemolytic antibody in the sera of the group receiving the standard dose of antigen. Similarly, hemolytic antibody was almost completely suppressed by cortisone acetate in the group receiving 10 times the standard antigen dose. In the groups receiving 50 and 100 times the standard amount of antigen, however, cortisone acetate did not prevent a substantial rise of hemolytic antibody although the levels were significantly below those of the controls. The data are illustrated in Fig. 4. This experiment was repeated with similar results.

Control Experiments

(*i*) *Effects of Cholesterol Blanks and the Cortisone Acetate Suspending Fluid on Production of Circulating Hemolytic Antibodies in the Mouse*

Two groups of 10 male and two groups of 10 female mice were injected subcutaneously with 0.1 ml. of a suspension containing 2.5 mgm. of cholesterol*, similar in particle size to the cortisone acetate used in these experiments and suspended in the same vehicle. Two other groups containing 10 animals of each sex received only the suspending fluid from which the cholesterol had been removed by centrifugation. Two female groups and one male group served as controls. The day following these injections all animals were given antigen; they were bled six days later.

Quantitative data on the hemolytic antibody levels of each pool of sera are given in Fig. 5, and show that neither cholesterol nor the suspending fluid had any significant influence on the normal rise of circulating hemolytic antibody levels.

* *Supplied by Merck and Company, Inc., Rahway, N.J.*

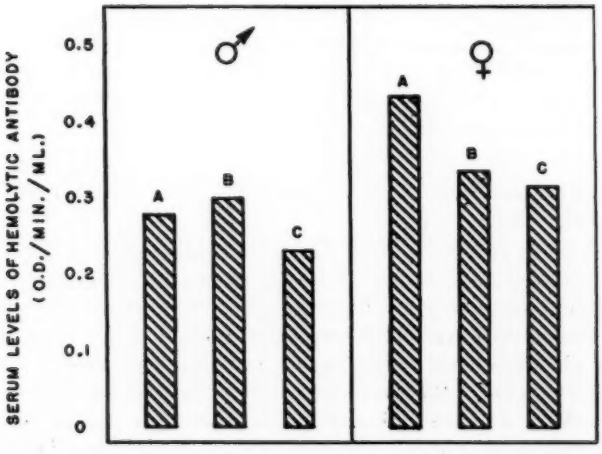

FIG. 5. Effect of cholesterol blanks and suspending fluid on circulating hemolytic antibody levels. A—control groups. B—groups receiving 2.5 mgm. cholesterol per mouse plus suspending fluid. C—groups receiving suspending fluid only.

TABLE IV

HEMOLYTIC ANTIBODY LEVELS OF SERA FROM NORMAL AND CORTISONE ACETATE TREATED MICE AND EFFECT OF SERA FROM CORTISONE ACETATE TREATED MICE ON THE DETERMINATION OF ANTIBODY LEVELS OF IMMUNE SERA

Source of serum	Sex	Dilution of serum in 5 ml. portion added to reaction mixture*	Hemolytic antibody level (O.D./min./ml.)
Non-immunized normal mice (groups of 10)	♂	1 : 10	0.00
	♂	1 : 10	0.00
	♀	1 : 10	0.00
	♀	1 : 10	0.00
Non-immunized cortisone acetate treated mice (groups of 10)	♂	1 : 10	0.00
	♂	1 : 10	0.00
	♀	1 : 10	0.00
	♀	1 : 10	0.00
Stock mouse antisera 206	♂	1 : 300	1.25
Stock mouse antisera 206 + serum from non-immunized cortisone acetate treated mice	♂	1 : 300 1 : 10	1.36
Stock mouse antisera 209	♀	1 : 300	1.12
Stock mouse antisera 209 + serum from non-immunized cortisone acetate treated mice	♀	1 : 300 1 : 10	1.15

* *Total volume of reaction mixture*—25 ml.

(ii) Effect of Sera from Non-immunized Cortisone Acetate Treated Mice on Hemolytic Reaction Rates

Each animal in two groups of 10 male and two groups of 10 female mice was injected subcutaneously with 2.5 mgm. of cortisone acetate on both day 1 and day 4 and bled on day 8. Four groups of 10 male and four groups of 10 female untreated mice were bled to provide normal sera. Each pool of the above sera when examined for hemolytic antibody was found to have a level of 0.0 O.D./min./ml. Portions of sera from the cortisone acetate treated mice were added in excess to hemolytic reaction mixtures containing mouse antisera to determine whether cortisone acetate could effect *in vivo* changes in the serum which might alter the characteristic velocity curves given by ordinary mouse antisera. As illustrated in Table IV, the sera from cortisone

acetate treated mice did not affect the normal velocity curves. The results of this experiment suggest that the observed effect of cortisone acetate in suppressing circulating immune hemolysins is due to a true absence of antibody and not to nonspecific factors which might have interfered with the *in vitro* hemolytic reactions.

Discussion

These experiments have demonstrated that a single subcutaneous injection of 2.5 mgm. of cortisone acetate in the male and female Swiss mouse will inhibit almost completely the normal production of circulating hemolytic antibody following an intravenous or intraperitoneal injection of 0.1 ml. of a 1.0% suspension of sheep erythrocytes. The results also show that the extent to which cortisone acetate suppresses circulating hemolytic antibody levels in the mouse is dependent upon the amount of antigen as well as the amount of hormone administered.

The biochemical mechanism of action of cortisone acetate in suppressing the appearance of antibody remains unknown. Such action may involve not only the antibody—its mechanism of formation, release to and disappearance from the blood, but also the antigen—its entry into metabolic reactions and the mechanism whereby it stimulates antibody production. A further complication to a rational concept of a mechanism at this time is the lack of experimental data proving that cortisone acetate itself and not one of its metabolites or some other substance whose production it stimulates is the biologically active agent causing the series of events that lead to the observed decrease in circulating antibody levels.

These experiments have provided a quantitative method for comparing the effect of various steroids, hormones, and other substances on circulating hemolytic antibody production. In such immunological procedures the variable antibody response of individuals makes it necessary to use large numbers of animals before significant data become available. For this reason, even with groups of 10 or 20 mice, it is necessary to draw conclusions cautiously and to rely only on profound differences in antibody levels. Arguments based on results showing minor variations in the concentrations of antibody above or below a "normal" level would not be convincing. Furthermore, since the reaction rates, used as a basis for determining antibody levels may vary with the red cell preparation, the same erythrocyte suspension is employed for the preparation of antigen as for the analysis of antibody. Also, appropriate control groups must be included with each experiment when comparisons are to be made of data obtained at different times. With these precautions in mind, however, the procedure has useful applications such as relating the antibody suppressive activity of different steroids on a quantitative basis. The method, when adequately controlled, also lends itself to a study of possible cortisone antagonists since a rise of antibody levels in animals receiving both cortisone and such antagonists would be significant.

Acknowledgments

This study was supported by a grant-in-aid from the Division of Medical Research of the National Research Council of Canada. The supplies of cortisone acetate were kindly donated by Dr. J. H. Laurie, Merck & Co. Ltd., Montreal, Canada. The technical assistance given by Miss Maureen Guild to some parts of the project is also gratefully acknowledged.

References

1. BJORNEBOE, M. D., FISCHEL, E. E., and STOERK, H. C. J. Exptl. Med. 93 : 37. 1951.
2. BOWMAN, W. M., MAYER, M. M., and RAPP, H. J. J. Exptl. Med. 94 : 87. 1951.
3. DEWS, P. B. and CODE, C. F. J. Immunol. 70 : 199. 1953.
4. GERMUTH, F. G., JR., OTTINGER, B., and OYAMA, J. Proc. Soc. Exptl. Biol. Med. 80 : 188. 1952.
5. GERMUTH, F. G., JR., OYAMA, J., and OTTINGER, B. J. Exptl. Med. 94 : 139. 1951.
6. KABAT, E. A. and MAYER, M. M. Experimental immunochemistry. Charles C. Thomas, Springfield, Illinois. 1948. p. 107.
7. MAYER, M. M., CROFT, C. C., and GRAY, M. M. J. Exptl. Med. 88 : 427. 1948.

INTRACELLULAR DISTRIBUTION OF PHOSPHOMONOESTERASES IN RAT LIVER HOMOGENATE[1]

By Claude Allard, Gaston de Lamirande[2], Hugo Faria[2, 3], and Antonio Cantero

Abstract

Acid phosphatase or phosphomonoesterase II activity of rat and mouse liver homogenates, prepared in 0.25 *M* sucrose, was found mainly in the cytoplasmic granules. Since the small percentage of activity of the nuclear fraction activity could be explained by the presence of mitochondria (which were actually counted in this fraction) it is concluded that rat and mouse liver nuclei do not contain acid phosphatase activity.

A rather broad range of acid phosphatase activity was observed in rat and mouse livers depending on the time elapsed between the preparation of homogenate and the activity determinations. However, a preincubation of the tissues or isolated fractions at 37° C. for 60 min. was sufficient to increase the activity to an optimal value, and thus eliminate variations due to the latency of this enzyme.

Alkaline phosphatase or phosphomonoesterase I activity was also found to be latent in rat liver homogenates. The phenomenon was less apparent than for acid phosphatase and seemed to depend mostly on the nature of the buffer employed in the assay system.

Some evidence for the presence of two forms of alkaline phosphatase in rat liver cells is presented. One form of the enzyme was found to have an absolute requirement of magnesium for activity and was present in the soluble fraction, whereas the other which was not activated by magnesium seemed firmly linked to the nuclei and microsomes and was absent in the soluble fraction. The activity in the mitochondrial fraction was small and seemed of doubtful significance.

Introduction

As a first step toward the study of phosphatases in experimental cancer of the liver, some general properties and the intracellular distribution of phosphomonoesterases I and II (or respectively, alkaline and acid phosphatase) in liver homogenate were investigated in the normal adult rat.

Recent studies on the intracellular distribution of acid phosphatase have been published (2, 12, 13). Palade (13), who investigated the distribution of this enzyme in purified homogenates and cytoplasmic fractions prepared in 0.88 *M* sucrose, observed that isolated nuclei contained a very low percentage of the total activity of the original homogenate. Few reports have appeared in the literature concerning the intracellular distribution of phosphomonoesterase I in liver homogenate. A study published in abstract form by Novikoff (11) and in greater detail recently (10, 12) showed that alkaline phenyl phosphatase, as measured in the presence of optimal concentration of magnesium ions, was mainly present in the supernatant fraction and in rather small amount in the particulate fractions. No conclusion was possible as to whether or not rat liver nuclei contained alkaline phosphatase activity. The results of Novikoff did not agree with those of Ludwig *et al.* (9).

[1] *Manuscript received in original form, November 5, 1953, and, as revised, March 15, 1954. Contribution from the Research Laboratories, Montreal Cancer Institute, Notre-Dame Hospital, and supported by a Grant-in-aid from the National Cancer Institute of Canada.*

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Materials and Methods

(1) Preparation of Tissue and Cellular Fractions

Wistar rats of 200–300 gm. were employed, taking no account of their feeding time. The animals were killed by decapitation and bled. The liver was then perfused with 40–50 ml. of ice-cold 0.25 *M* sucrose by way of the portal vein. Frequent massage of the liver during this process assured a nearly blood-free liver. The liver was then excised, chilled on ice, blotted on filter paper, and weighed. A liver pulp was prepared by forcing the whole liver through an all plexiglass squeezer. The connective tissue was discarded and an exact amount of liver pulp was quickly weighed in order that a 10% homogenate in 0.25 *M* sucrose could be prepared. This was carried on, with a smooth plastic pestle turning at 600 r.p.m. for two minutes. The number of whole cells, as determined by actual counts, was below one per cent with this comparatively new homogenizer*.

Liver fractions, namely, nuclear (N), mitochondrial (M), microsomal (Mc), and supernatant (S) were obtained by centrifugation of homogenates prepared in 0.25 *M* sucrose, as described by Schneider *et al.* (16). The supernatant fraction contained the washings of all isolated fractions. A Servall SS-1 and a refrigerated Spinco Model L centrifuge were employed. For sonic vibration studies, Raytheon apparatus model R. (9 kilocycles) was employed at vibration intensity of 175 v.

(2) Substrate, Buffers, and Reagents

Sodium beta-glycerophosphate (Eastman Kodak, Rochester, N.Y.) was the sole substrate employed in the present phosphatase studies.

The buffers were either acetate–acetic acid, acetate–borate–cacodylate (ABC; cf. (4)), sodium barbital (veronal), or 2-amino-2-methyl-1,3-propanediol (ammediol) (5). These chemicals and others not mentioned here were the Chemically Pure grade, except ammediol which was the practical grade available from Eastman Kodak, Rochester, N.Y.

(3) General Procedure for Enzyme Assay

The enzyme assay was conducted in vials of 9 by 2.2 cm., at 37° C. ± 0.5° C. in a Warburg bath which had been modified to hold three metal supports of 40 vials each. Each vial was corked and constantly agitated during the enzymatic reaction, which lasted 30 or 60 min.

Prior to the addition of homogenate or cellular fraction suspension, the buffered substrate was maintained at 37° C. for 10 min. The volume of homogenates and fractions was between 0.1 to 2 ml. according to their respective activity. In all experiments the final volume in the reaction vessel was 6 ml. Whenever possible, the reaction was conducted at two levels of tissue. The concentrations of freshly prepared substrate and buffer were so arranged that two volumes of substrate with two volumes of buffer gave the desired final concentration. The reported pH's were those measured with a Beckman pH Meter model G, after enzymatic reaction.

* *Available from A. H. Thomas Co., Philadelphia, U.S.A.*

The reaction was stopped by the addition of 6 ml. of 10% trichloroacetic acid (TCA). Contents of the vials, transferred to centrifuge tubes, were centrifuged at 3500 r.p.m. for 10–15 min. This procedure was preferred to filtration and found to be as accurate. The phosphorus analyses were conducted on 3 ml. of the TCA supernatant by the method of Fiske and Subbarow. All colorimetric readings were done with a Beckman Quartz spectrophotometer model DU in glass cells No. 2097 at 450 mμ. Controls were run in each experiment for inorganic phosphate in the substrate-buffer mixtures, as well as in liver homogenates and fractions.

Results

(*1*) *Phosphomonoesterase II*

In preliminary work a system recently described by Palade (13) was employed for the investigation of acid phosphatase activity as a function of pH. Such determinations were made in the homogenate and isolated fractions because it seemed inappropriate to assume the same optimal pH of activity in each separate fraction isolated by centrifugation*· †. Owing to a different environment the enzyme might behave differently. However, with acid phosphatase, maximal pH of activity was observed between 4.3 and 4.5 in homogenates and in all the isolated fractions, whether acetate–acetic or acetate–borate–cacodylate (ABC, cf. (4)) buffers were used. The pH-activity curves were established with an optimal final concentration of substrate (0.08 *M*).

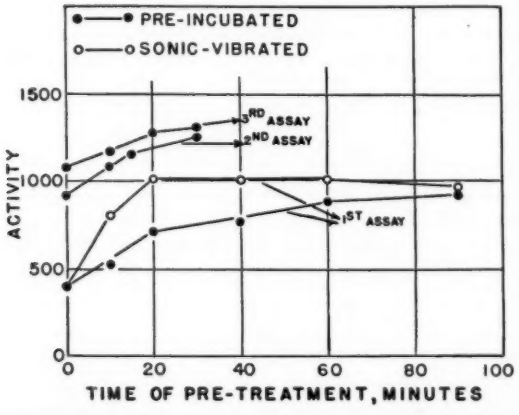


FIG. 1. Effect of preincubation and sonic vibration on phosphomonoesterase II activity of rat liver homogenates. The latter were prepared in 0.25 *M* sucrose and either sonic vibrated at 5° C. in a Raytheon apparatus or preincubated at 37° C. and aliquots were sampled at various intervals. Acid phosphatase activity, which was determined in a system described by Palade (14), is expressed in gamma phosphorus liberated during 60 min. at 37° C. per 100 mgm. of fresh tissue at final pH 4.5.

* *The optimal pH of activity of desoxyribonucleodepolymerase is different in the mitochondrial (pH 5.1) fraction as compared to the soluble fraction (pH 6.6). (Unpublished data.)*

† *Ribonucleodepolymerase possess an acid and alkaline peak of activity in rat liver homogenates.* (Lamirande, G. De, *et al.* Science, 119 : 351. 1954.)

During the course of this work, acid phosphatase activity of homogenate and particulates was repeatedly found to be enhanced by aging at 5° C. Since latent acid phosphatase is known to be present in the mitochondrial and microsomal fractions (2), it was suspected that 10 min. of preincubation at 37° C. (as in Palade's procedure (13)) was not sufficient to develop full activity. Fig. 1 shows, in fact, that 30–60 min. were necessary. In one of these experiments, the efficiency of preincubation at 37° C. was compared with the effect of sonic vibrations, which are known to break a high percentage of cytoplasmic granules (7). It was evident that with sonic waves the activity was more rapidly enhanced, but was comparable to a 60 min. preincubation at 37° C. Since our main interests lie in the total acid phosphatase of liver as compared to pathological liver, the distribution of this enzyme was studied in preincubated fractions, once their isolation by centrifugation was completed.

Table I shows the intracellular distribution of the total phosphomonoesterase II activity in adult rat liver homogenates prepared in 0.25 *M* sucrose.

TABLE I

INTRACELLULAR DISTRIBUTION OF PHOSPHOMONOESTERASE II ACTIVITY* IN RAT AND MOUSE LIVER HOMOGENATES

Fresh homogenate	Sixty minutes preincubation at 37 ± 0.5° C.						Number of experiments
	Homogenate	(N)	(M)	(Mc)	(S)	Recovery	
(1) Activities of rat liver fresh homogenates and of preincubated fractions and homogenates							
846 ± 104.7	1054 ± 105.4	65.7 ± 31.3	585 ± 57.6	348 ± 49.4	79 ± 25.8	— —	— —
Percentage distribution		6.1 ± 2.5	55.6 ± 1.4	33.0 ± 3.1	33.0 ± 3.1	102.8 ± 2.3	4, 8 animals
(2) The nuclear and mitochondrial fractions have been corrected for the activity of the mitochondria present in the nuclear fraction, by actual counts of mitochondria (1) in the respective liver fractions							
—	1294	22.5	715	492	143	—	—
Percentage distribution		(1.7)	(55.2)	(38.0)	(11.0)	(105.9)	2, 4 animals
(3) Mean intracellular distribution in mouse liver. Corrections as described in part (2) of this table have been made							
367	483	5	244	190	67.9	—	—
Percentage distribution		(1.0)	(50.5)	(39.3)	(14.0)	(104.8)	2, 8 animals

* *Activity is expressed in gamma of phosphorus liberated from 0.08 M beta-glycerophosphate, per 100 mgm. of fresh tissue or its equivalent at 37° C. ± 5° C. and at a final pH of 4.5.*

The greatest percentage of activity was found mainly in the cytoplasmic granules whereas the nuclear and supernatant fractions showed very little activity. Similar distribution has been observed previously (2, 3, 13). The low activity of the nuclear fraction can be attributed mostly to some mitochondria contamination. Table I, part 2, illustrates that when correction of the nuclear and mitochondrial fractions activities were made with respect to the number of mitochondria actually present in these fractions, a very slight activity could then be assigned to the nuclei. The mitochondria were counted as described previously (1). In mouse liver, where the experimental conditions were the same as for rat liver, the distribution appeared similar (Table I, part 3) though the total activity was about half that of rat liver. Latency of acid phosphatase was also observed.

(2) *Phosphomonoesterase I*

In a system similar to the one used for acid phosphatase, the optimal pH of liver homogenate and fractions phosphatase activity was tested over a range of pH 5.0 to 10.5 at 0.2 pH intervals, in the presence or absence of magnesium ions as activator. The pH-curves of activity were obtained from measurements made in ABC, veronal, and ammediol buffers at the respective final concentration of 9.9×10^{-4} *M*, 2.4×10^{-2} *M*, and 5.0×10^{-2} *M*. Magnesium chloride was used at the final optimal (see Table II) concentration of 10^{-2} *M*. The final concentration of substrate was 0.1 *M*, which was found to be optimal. Separate determinations have shown that the alkaline phosphatase activity was proportional to incubation time (0 to 60 min.) and to the concentrations of tissue. Analysis of the pH curves of activity has revealed that a peak of activity was at pH 9.6 in ABC buffer with or without Mg; at 9.2 in veronal or ammediol *when Mg was present*, and a peak at 9.8 in the *absence of Mg*. Displacement of optimal pH by Mg has been observed with purified preparation of alkaline phosphatase (6, 14), and Gomori stressed the importance of this fact in the study of enzyme activators (6).

A systematic study of magnesium activation of each separate fraction isolated from liver homogenate showed the nuclear, mitochondrial, and microsomal fractions to be slightly activated by this ion as compared with the supernatant fraction. This phenomenon was more apparent when the activity was measured in ammediol buffer at the optimal pH of 9.2 in the presence of Mg, and 9.8 in absence of Mg (Table II).

The possibility that the small enzymatic activities observed in the absence of Mg were effectively due to magnesium actually present in the particulates was tested on dialyzed (N), (M), and (Mc) fractions. Dialysis was conducted at 5° C., in viscose tubing against infinite volume of 0.25 *M* sucrose for 70 hr.; this was found more than sufficient to remove Mg ions added to the soluble fraction, at a final concentration of 0.01 *M*. Table III shows that dialysis of the soluble fraction (control) to which Mg was added resulted in the total loss of activity, whereas the particulate activity was not significantly affected.

TABLE II

EFFECT OF MG ON PHOSPHOMONOESTERASE I ACTIVITY* LINKED TO LIVER FRACTIONS

Final concentration of Mg $M \times 10^2$	Liver Fractions															
	Nuclear				Mitochondrial				Microsomes				Supernatant			
	Buffers															
	ABC	Veronal	Ammediol		ABC	Veronal	Ammediol		ABC	Veronal	Ammediol		ABC	Veronal	Ammediol	
	Final pH															
	9.6	9.2	9.2	9.8	9.6	9.2	9.2	9.8	9.6	9.2	9.2	9.8	9.6	9.2	9.2	9.8
0.00	20	(4)† 15 ± 3		(3) 41 ± 5	11	(3) 22 ± 6		(4) 21 ± 6	42	30		(3) 46 ± 7	0	0		(4) 8 ± 7
0.25	19	30			18	25			50	39			98	—		
0.50	24	32			21	28			54	43			137	227		
1.00	25	(4) 27 ± 5	(3) 37 ± 4		23	(3) 38 ± 6	(4) 33 ± 4		53	42	(3) 49 ± 13		157	250	(4) 274 ± 32	
1.50	22	—			21	—			54	—			146	—		
2.00	21	31			21	31			48	43			121	227		
2.50	21				21				47				115			
3.00	17				19				46				107			

* *Activity is expressed in gamma of phosphorus liberated from 0.1 M beta-glycerophosphate per hour, per an equivalent of 100 mgm. of tissue at 37 ± 0.5° C.*

† *Number of experiments with standard deviation below activity value.*

TALBE III

EFFECT OF MG ON ENZYME ACTIVITY* DIALYZED LIVER FRACTIONS

Liver fractions	Final Mg concentration in the system			
	0.00 *M*	0.01 *M*	0.00 *M*	0.01 *M*
	Before dialysis		After dialysis	
(N)	21.4	35.7	13.8	32.4
(M)	10.5	16.7	11.4	25.2
(Mc)	21.4	26.6	18.1	27.1
(S)	0	305	0	255
Control: (S), to which Mg was added to a final concentration of 0.01 *M* (see text).	—	324	0	—

* *Determined in veronal buffer (2.4×10^{-2} M) and beta-glycerophosphate 0.1 M, at final pH 9.2. Activity is expressed as in Table II.*

It was verified that the addition of (N), (M), and (Mc) fractions, when alkaline phosphatase has been inactivated by heat treatment (30 min. at 85° C.), did not activate the supernatant fraction activity in the absence of added Mg.

The above information, and also the fact that the supernatant contained the washings of all particulate fractions and still did not possess activity in the absence of Mg, suggested that the alkaline phosphatase complex is firmly bound to cellular elements or that cellular Mg is preferentially adsorbed and strongly bound to particulates.

The following experiments were, however, devised to determine whether the alkaline phosphatase linked to these cellular elements could be liberated and become effectively active in soluble form. Sonic vibrations were employed as a mean to break the particulates and liberate the enzyme (cf. (7)). Table IV shows that sonic vibration had no effect on the alkaline phosphatase activity (with or without Mg added) but effectively liberated part of the particulate enzyme (58%) into solution. Dialysis of the sonic treated homogenate did not alter the phosphatase activity (Table IV, part 3). These results suggest that particulate alkaline phosphatase might be different from the soluble fraction phosphatase, since it is not activated by Mg, even when brought into solution.

The fact that sonic treatment did not enhance alkaline phosphatase activity as measured in veronal buffer at pH 9.2 is not in accordance with increase of activity noted when ammediol buffer was employed in the study of the action of aging or heat (Fig. 2). Such a discrepancy appears to be due to the nature of the buffer employed in the assay system. Table V shows, in fact, that ammediol might be superior to veronal for preserving the alkaline phosphatase complex during the enzymatic reaction. It is known that veronal does damage cytoplasmic particles (15).

TABLE IV

EFFECT OF SONIC VIBRATION ON PHOSPHOMONOESTERASE I ACTIVITY*

Liver fractions	Activity before sonic treatment		Activity after sonic treatment	
	Mg final concentration			
	0.01 *M*	0.00 *M*	0.01 *M*	0.00 *M*
Part 1. Homogenates and fractions were sonic vibrated during 30 min. at 5° C.				
Homogenate	352.0	78.0	350.0	78.1
Nuclear	30.8	15.7	30.8	18.1
Mitochondrial	36.1	21.2	39.5	21.4
Microsome	54.0	35.2	51.4	33.8
Supernatant	212.0	0	229.0	0
Recovery (%)	94.6	92.3	100.0	93.8
Part 2. Homogenate, the once-washed sediment (obtained by centrifugation of the homogenate at 140,000 × g. during one hour), which contained (N), (M), and (Mc), and the sediment supernatant and washings were sonic vibrated as above				
Homogenate	—	79.0	350.0	79.0
Sediment	—	75.8	41.4	31.6
Supernatant and washings	—	0	304.0	48.2
Recovery (%)	—	95.9	98.6	101.0
Part 3. Sonic treated homogenate dialyzed 85 hr. against 0.25 M sucrose at 5° C.				
			360.0	81.9

* *Expressed as in Table III.*

TABLE V

EFFECT OF PREINCUBATION (60 MIN. AT 37 ± 0.5° C.) ON LIVER HOMOGENATE ACTIVITY AS MEASURED IN DIFFERENT BUFFERS

Fresh homogenate		Preincubated homogenate	
Mg final concentration			
0.0 *M*	0.01 *M*	0.0 *M*	0.01 *M*
(*1*) *Ammediol buffer 0.025 M; pH 9.8 without Mg and 9.2 with Mg*			
96.2	314	175	371
78.1	381	168	444
(*2*) *Veronal buffer* 2.4×10^{-2} *M; pH 9.2*			
—	425	—	444
—	376	—	405

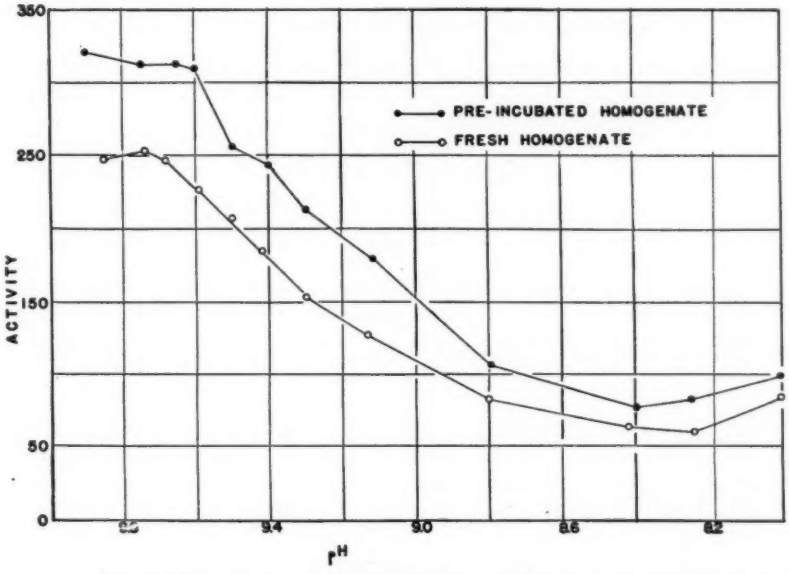


FIG. 2. pH-activity curves of phosphomonoesterase I in fresh and preincubated homogenates (60 min. at 37° C.) were compared. Activity is expressed in gamma phosphorus liberated from 0.1 *M* beta-glycerophosphate per 100 mgm. of fresh tissue at 37 ± 0.5° C. during 60 min. Determinations were made in the absence of Mg, and at constant concentrations of buffer ions (ammediol).

Table VI shows the intracellular distribution of phosphomonoesterase I activity in rat liver homogenate. In the first series of experiments (Table VI, parts 1, 2, 3, 4) the determinations were made in different buffers and in the presence of optimal concentrations of magnesium whereas in a second series (parts 5, 6) the activity was determined in the absence of Mg.

In the presence of Mg, the phosphatase activity and percentage distribution as measured in veronal and ammediol buffers were similar. The supernatant contained about 70% of the whole homogenate activity, whereas the residual activity was about equally distributed in the other cellular fractions. In ABC buffer however, the homogenate activity was much lower than in veronal or ammediol buffers. This low activity is attributed to specific inhibition of the supernatant fraction by the ABC buffer (Table VI, part 2). It is of interest to mention that no such inhibition was observed in the cellular fractions. Borate and cacodylate ions have repeatedly been shown to inhibit alkaline phosphatase (17).

In the absence of Mg, the supernatant did not show any activity whereas the nuclear and microsomal fractions each contained about 40% of the original homogenate and the mitochondrial fraction 15%.

TABLE VI

INTRACELLULAR DISTRIBUTION OF PHOSPHOMONOESTERASE I ACTIVITY* IN RAT LIVER HOMOGENATE

Homogenate	(N)	(M)	(Mc)	(S)	Recovery	Number of experiments
(1) Veronal buffer, 2.4 × 10^{-2} M in the presence of Mg, (0.01M), final pH: 9.2						
418 ± 56.6	27.7 ± 11.5	31.7 ± 8.5	49.7 ± 15.1	302.6 ± 52.0		
Percentage distribution	6.6 ± 2.1	7.8 ± 2.0	11.9 ± 3.1	72.5 ± 9.0	98.9 ± 5.8	8, 16 animals
(2) ABC buffer, 9.9 ± 10^{-4} M in the presence of Mg, (0.01 M), final pH: 9.6						
297 ± 82.5	41.5 ± 19.0	22.4 ± 8.3	65.1 ± 16.4	171.8 ± 57.8		
Percentage distribution	13.4 ± 2.8	7.6 ± 2.3	23.3 ± 9.4	56.3 ± 11.0	101.6 ± 6.9	6, 12 animals
(3) Ammediol buffer, 0.025 M in the presence of Mg (0.01 M), final pH: 9.2						
398 ± 51.4	41.3 ± 5.8	33.6 ± 4.3	48.2 ± 12.9	280.7 ± 39.8		
Percentage distribution	10.4 ± 1.0	8.4 ± 0.2	11.9 ± 2.0	70.6 ± 7.6	101.4 ± 5.3	3, 6 animals
(4) Same experimental conditions as 3, but preincubated 60 min. at 37° C.						
421 ± 61.9	59.0 ± 10.8	33.1 ± 5.3	55.2 ± 9.1	265.8 ± 26.4		
Percentage distribution	14.9 ± 3.0	8.1 ± 1.0	13.7 ± 1.5	66.3 ± 7.0	101.4 ± 5.3	5, 10 animals
(5) Ammediol buffer, final pH 9.8 in the absence of Mg						
112.3 ± 13.8	40.8 ± 5.0	18.5 ± 3.7	46.6 ± 5.7	10.2 ± 7.9		
Percentage distribution	36.9 ± 6.5	16.8 ± 4.1	41.8 ± 4.6	8.3 ± 6.4	103.8 ± 5.0	3, 6 animals
(6) Ammediol buffer, final pH 9.8 in the absence of Mg, but preincubated 60 min. at 37° C.						
187.6 ± 48.9	74.1 ± 20.9	30.5 ± 8.4	83.4 ± 21.1	7.3 ± 7.8		
Percentage distribution	37.4 ± 0.9	15.4 ± 1.1	42.6 ± 6.4	3.3 ± 2.9	103.8 ± 5.0	5, 10 animals

** Activity is expressed in gamma phosphorus liberated from 0.1 M beta-glycerophosphate per 100 mgm. of fresh tissue or its equivalent, per hour, at 37 ± 0.5° C.*

Discussion

Phosphomonoesterase II

The acid phosphatase activity of rat liver homogenate prepared in 0.25 *M* sucrose has been shown to increase to a maximum activity after 30 to 60 min. of preincubation at 37° C. These results suggest that the substrate (sodium beta-glycerophosphate) is readily available to acid phosphatase linked to cellular granules only after these particulates have been severely damaged. Therefore depending on the procedure employed a rather broad range of acid phosphatase activity might be found in rat liver and possibly in other organs. As pointed out by de Duve *et al.*, "free" acid phosphatase (2) was measured when conditions of isolation as well as the biochemical determinations of the enzymatic activity were such as to respect what seemed to be the membrane of the granules. However, when the cellular units were broken with the aid of the Waring blendor, or by other means, the total acid phosphatase activity could be determined (2). Since breaking techniques are difficult to control, it was preferred to preincubate the homogenate or isolated fractions during periods sufficient to measure maximum available enzymatic activity. Such procedure has the advantage of being easily reproduced, which is a prerequisite in comparative studies and in pathological biochemistry. In intracellular distribution studies it seems important to measure the total enzymic activity, since latency of activity in a preferential particulate might greatly affect the percentage distribution.

Our results on the intracellular distribution of acid phosphatase have confirmed and extended the work of others (2, 13) that rat and mouse liver nuclei did not contain activity whereas the two general classes of granules namely, the mitochondria and microsomes were the site of most of the homogenate activity. The nucleus has been shown to be free of activity by the indirect method of counting the mitochondria in the nuclear and mitochondrial fractions. This method of studying the nuclear fraction enzymes seemed less tedious and more simple than to prepare pure nuclei, by present available techniques (8).

Phosphomonoesterase I

Study of the effect of magnesium ion on the isolated fractions of liver homogenate has revealed that this ion slightly activates the alkaline phosphatase linked to particulates, whereas the soluble fraction was greatly activated. Furthermore, the soluble fraction was shown to have an absolute requirement of magnesium ion for activity. These two different states of metal activation of alkaline phosphatase, and also the fact that ABC buffer inhibited only the soluble fraction activity, might indicate the presence of two forms of phosphatase within the liver cells. An hypothesis which is being considered is that alkaline phosphatase is "active" (or metal activated) *in situ* in the nuclei and microsomes and "inactive" in the cellular sap. As for the mitochondria they probably contain no activity. The nature of the "inactive" form is presently unknown but may be similar to the inactive

phosphatase obtained after dialysis of purified extract of this enzyme, which is known to have an absolute requirement of magnesium for activity (14). The ratio of "inactive"/"active" phosphatase appears variable in pathological conditions of the liver as well as in other tissues. In intestine, kidney and in liver tumor the enzyme is in a high native "activated state" and mainly found in the microsome fraction.*

The intracellular distribution of phosphomonoesterase I in rat liver, when measured with an optimal concentration of magnesium, was found similar to phenyl phosphatase activity reported by Novikoff (10) but did not confirm results of other investigators (9). Such discrepancies might be due to the use of different buffers and also the slight differences in the centrifugation procedures.

Acknowledgments

The technical assistance of Miss Denise Plante is gratefully acknowledged.

References

1. ALLARD, C., MATHIEU, R., LAMIRANDE, G. DE, and CANTERO, A. Cancer Research, 12 : 407. 1952.
2. BERTHET, J. and DUVE, C. DE. Biochem. J. 50 : 174. 1951.
3. DUVE, C. DE Exposés ann. biochim. méd. 14 : 47-72. 1952.
4. DUVE, C. DE., BERTHET, J., HERS, H. G., and DUPRET, L. Bull. soc. chim. biol. 31 : 1242. 1949.
5. GOMORI, G. Proc. Soc. Exptl. Biol. Med. 62 : 33. 1946.
6. GOMORI, G. Biochim. et Biophys. Acta, 8 : 162. 1952.
7. HOGEBOOM, G. H. and SCHNEIDER, W. C. Nature, 166 : 302. 1950.
8. LAMIRANDE, G. DE, ALLARD, C., DA COSTA, H., and CANTERO, A. Can. J. Biochem. Physiol. 32 : 35. 1954.
9. LUDWIG, S. and CHANUTIN, A. Arch. Biochem. 29 : 441. 1950.
10. NOVIKOFF, A. B. Exptl. Cell Research, Suppl. II. 1952.
11. NOVIKOFF, A. B., PODBER, E., and RYAN, J. Federation Proc. 9 : 210. 1950.
12. NOVIKOFF, A. B., PODBER, E., RYAN, J., and NOE, E. J. Histochem. Cytochem. 1 : 27 1953.
13. PALADE, G. E. Arch. Biochem. 30 : 144. 1951.
14. ROCHE, J. *In* The enzymes. Vol. I. Part I. Academic Press Inc., New York. 1950.
15. SCHNEIDER, W. C. J. Histochem. Cytochem. 1 : 212. 1953.
16. SCHNEIDER, W. C. and HOGEBOOM, G. H. J. Biol. Chem. 183 : 123. 1950.
17. ZITTLE, C. A. and DELLA MONICA, E. S. Arch. Biochem. 26 : 112. 1950.

* *Unpublished data.*

TISSUE GLYCOGEN AND GLUCOSE ABSORPTION IN RATS ADAPTED TO COLD[1]

By Edouard Pagé and Louis-Marie Babineau

Abstract

Fasted rats previously fed a high fat ration and adapted to cold maintain their liver glycogen as efficiently as their controls kept at room temperature. On a high carbohydrate diet, fasting liver glycogen is markedly higher in the cold adapted animals. Glucose absorption rate on the high fat regime is nearly 70% higher following adaptation to cold.

Introduction

Rats adapted to cold show a lower respiratory quotient than nonadapted ones following a 26 hr. fast at room temperature. Following the administration of maltose, however, the rise in R.Q. is always greater in the adapted animals irrespective of the diet and of the temperature at which the determination is made (10). The present experiments were undertaken to find out (*a*) whether fasted rats adapted to cold burn more fat preferentially or as a matter of necessity consequent to a more exhaustive depletion of their glycogen stores during fast than nonadapted rats; and (*b*) whether the faster rate of utilization of administered maltose in these adapted animals merely reflects an accelerated rate of glucose absorption.

Experimental

The experimental rations were the same as described previously (10) with the exception of the casein content which was raised to 18% on the low fat ration and to 29% (by weight) on the high fat ration, both levels being the same on a dietary caloric basis. Fifteen rats were put on the low fat diet and 45 on the high fat ration at each ambient temperature. The animals were housed in individual metal cages and were given food and water ad libitum. The average body weight for each group was 223 gm. initially. Temperature in the cold room varied between 8° and 13° C. during the first seven days of the experimental period; it rose to nearly room temperature for the following week owing to mechanical failure and was lowered to between 5° and 7° C. for the balance of the period.

Fasting glycogen values were determined in all groups between the 63rd and 79th day and glucose absorption, between the 83rd and 99th day. All rats were fasted at room temperature from 10 o'clock in the morning until the same time on the next day, when they were sacrificed by decapitation a few minutes after receiving a massive dose (65 mgm.) of Nembutal intraperitoneally. This barbiturate has been reported to have no effect on intestinal

[1] *Manuscript received March 8, 1954.*

Contribution from the Département de physiologie de la nutrition, Faculté de Médecine, Université Laval, Québec, P.Q., with financial assistance from the Defence Research Board of Canada (Grant No. DRB-141).

movement, at least over the period concerned (6), nor should it affect tissue glycogen significantly (11). Glucose absorption was measured in similarly fasted rats. The animals received one cubic centimeter of a 50% glucose solution per 100 gm. of body weight. This was administered between 9 and 10 o'clock for the one hour absorption period and between 11 and 12 o'clock for the three hour period. The glucose absorption was measured by the Cori (2) method, with two small modifications: the glucose was administered through a No. 8 catheter connected to a microburette as suggested by MacKay and Bergman (7); positive pressure was applied to the surface of the solution by means of an inflated rubber bladder and tubing in order to hasten delivery. The intestinal washings were collected in a 500 cc. volumetric flask. They were freed of interfering substances by the successive addition of 50 cc. of 0.3 *N* barium hydroxide and 50 cc. of a 5% zinc sulphate solution.

Liver glycogen was determined on the whole livers and muscle glycogen on the gastrocnemius. The glycogen was precipitated by the method of Good *et al.* (5). The glucose was measured titrimetrically in liver glycogen hydrolyzates and in intestinal contents by the method of Shaffer and Somogyi (13). Nelson's method (8) was used for the muscle glycogen.

Results

Changes in body weight with environmental temperature and diet are shown in Table I. It is noted that in spite of the large cellulose content of the high fat diet, the animals in these groups nevertheless made slightly larger gains than the ones on the low fat diet.

TABLE I

AVERAGE GAINS IN BODY WEIGHTS IN GRAMS AFTER 62 DAYS ON THE EXPERIMENTAL RATIONS

	Room temperature	*P**	Cold room
High fat group	204 ± 7.31**	< .01	174 ± 4.93
P	.05	—	< .01
Low fat group	183 ± 5.60	< .01	151 ± 5.44

* *P = Probability of identity between the numbers on either side.*
** *Standard error of the mean.*

Table II shows the liver weights and the glycogen contents of the liver and muscle of the fasted rats. As we had noted previously (9), livers of animals exposed to cold are larger and more so on the low fat diet.

When comparing diets at room temperature, our data are in full accord with the findings of Samuels *et al.* (12); the animals on the high fat diet spare their liver glycogen to a greater extent, presumably through a preferential utilization of lipids. In the cold, the liver glycogen concentration of the high

TABLE II

EFFECT OF ADAPTATION TO COLD ON FASTING GLYCOGEN VALUES IN RATS FED A HIGH OR A LOW FAT RATION

	High fat ration			Low fat ration		
	Room temperature	*P*	Cold room	Room temperature	*P*	Cold room
Number of rats	15		15	15		14
Body weight (gm.)	323		306	319		278
Liver weight gm./100 gm. B.W.	2.39 ± 0.04	< .01	2.78 ± 0.04	2.45 ± 0.07	< .01	3.10 ± 0.06
Liver glycogen mgm./100 gm. liver	857 ± 112	> .05	619 ± 61	138 ± 26	< .01	330 ± 56
mgm./100 gm. B.W.	20.4 ± 2.75	> .4	17.8 ± 1.72	3.4 ± 0.67	< .01	10.4 ± 1.84
Muscle glycogen mgm./100 gm. muscle	509 ± 22	> .2	557 ± 32	564 ± 32	> .6	581 ± 22

TABLE III

EFFECT OF ADAPTATION TO COLD ON GLUCOSE ABSORPTION IN RATS FED A HIGH FAT RATION AND AVERAGE BODY WEIGHTS OF THE ANIMALS AT THE TIME OF THE DETERMINATIONS

	Room temperature animals	*P*	Cold room animals
Glucose absorption (mgm./100 gm. B.W./hr.)			
One hour period	88 ± 5.53	< .01	140 ± 13.11
Three hour period	82 ± 8.18	< .01	146 ± 7.13
Combined	85 ± 4.73	< .01	143 ± 6.99
Body weight (gm.)			
One hour absorption	(10)* 395 ± 11.3	< .02	(11) 344 ± 10.2
Three hour absorption	(10) 381 ± 7.4	< .01	(11) 299 ± 9.5
Combined	(20) 388 ± 6.8	< .01	(22) 322 ± 8.4

* *Number of animals per group.*

fat group does not differ significantly from that of their controls. On the low fat ration, however, there occurs a very marked rise in the fasting glycogen value, as compared to the low fat group at room temperature.

Neither diet nor environmental temperature seem to affect muscle glycogen significantly; this confirms earlier data of Baker and Sellers (1).

Glucose absorption rates of animals on a high fat diet are shown in Table III. At a given temperature, there is a very close agreement between the rate measured after one and three hours of absorption, respectively. At room temperature, the absorption rate is low and of the same order as reported by Sinclair and Fassina (14) for animals on a high fat diet. In the cold, the absorption rate is very much higher. That this is not due simply to the smaller size of the animals is indicated by the fact that absorption rate at one and three hours is the same in the cold in spite of the fairly large difference in body weight of the two groups. It is also recalled that the amount of glucose given was proportional to body weight.

The deposition of glycogen in the liver and muscle tissue following glucose absorption is shown in Table IV. In the liver, the deposition is insignificant after one hour of absorption. At three hours, it is of the same order as reported by Cori (3) for similarly low amounts of absorbed glucose. These amounts are too low to show up any effect of cold on liver glycogen deposition, if there is such an effect.

Glucose uptake by muscle tissue is apparently much more rapid. There occurs a significant rise in muscle glycogen after one hour. At three hours, the glycogen content is not significantly higher than after one hour. It is surmised that the tissue is already approaching its prefasting glycogen concentration.

TABLE IV

LIVER AND MUSCLE GLYCOGEN FOLLOWING GLUCOSE ABSORPTION

	Room temperature			Cold room		
	One hour absorption	*P*	Three hour absorption	One hour absorption	*P*	Three hours absorption
Liver glycogen						
mgm./100 gm. liver*	950 ± 95	< .01	1379 ± 168	651 ± 119	< .01	1791 ± 257
mgm./100 gm. B.W.*	21.0 ± 2.19	< .02	34.1 ± 4.09	18.0 ± 3.32	< .01	52.7 ± 7.63
% Glucose absorbed deposited as liver glycogen	2.4		5.2	0.6		7.8
Muscle glycogen* mgm./100 gm.	612 ± 42	0.6	649 ± 52	657 ± 19	0.2	742 ± 60

* *See Table II for corresponding fasting values.*

Discussion and Conclusions

It is fairly clear from the above data that in cold adapted animals fasted for one day, at room temperature, the glycogen content of the liver is as high as in animals not adapted to cold when both groups are fed a high fat diet. On a low fat diet, however, liver glycogen is actually higher in the cold adapted rats. It would thus seem that adaptation to cold has a sparing effect on liver glycogen comparable in nature, if not in magnitude, to that resulting from the feeding of a high fat diet. It may further be concluded that the lower fasting R.Q.'s of cold adapted rats are indicative of a preferential utilization of fat, since their liver and muscle glycogen reserves were as high or higher than those of the nonadapted controls at the time the measurements were made.

The considerable rise of the glucose absorption rate in rats adapted to cold is not surprising in view of the larger load imposed on absorptive processes by the increased food intake and of the general increase in metabolic rate for various other tissues studied to date (4, 15). It would be interesting to know the contribution of the intestinal tract to extra heat production in the cold. These data, on glucose absorption, supply a ready explanation to the higher R.Q.'s of cold adapted rats in the course of glucose absorption; it is clear that these animals had to dispose of a larger amount of glucose during the time interval required for R.Q. determination. Whether the extra glucose was being oxidized or transformed into fat cannot be stated. From certain analogies between adaptation to cold and to high fat diets: higher fasting liver glycogen, lower fasting R.Q.'s, and lower liver glycogen in the nonfasting state in both situations (1, 12), one is inclined to believe that rats adapted to cold burn fat preferentially and that an increased rate of lipogenesis may result therefrom.

References

1. BAKER, D. G. and SELLERS, E. A. Am. J. Physiol. 174 : 459. 1953.
2. CORI, C. F. J. Biol. Chem. 66 : 691. 1925.
3. CORI, C. F. J. Biol. Chem. 70 : 577. 1926.
4. DESMARAIS, A. Rev. can. biol. 12 : 99. 1953.
5. GOOD, C., Kramer, H., and SOMOGYI, M. J. Biol. Chem. 100 : 485. 1933.
6. GOODMAN, R. D., LEWIS, A. E., SCHUCK, E. A., and GREENFIELD, M. A. Am. J. Physiol. 169 : 236. 1952.
7. MACKAY, E. M. and BERGMAN, H. C. J. Biol. Chem. 101 : 453. 1933.
8. NELSON, N. J. Biol. Chem. 153 : 375. 1944.
9. PAGÉ, E. and BABINEAU, L. M. Can. J. Med. Sci. 31 : 22. 1953.
10. PAGÉ, E. and CHÉNIER, L. P. Rev. can. biol. 12 : 530. 1953.
11. REINDOLLAR, W. F. Proc. Soc. Exptl. Biol. Med. 33 : 182. 1935.
12. SAMUELS, L. T., REINECKE, R. M., and BALL, J. H. A. Proc. Soc. Exptl. Biol. Med. 49 : 456. 1942.
13. SHAFFER, P. A. and SOMOGYI, M. J. Biol. Chem. 100 : 695. 1933.
14. SINCLAIR, R. G. and FASSINA, R. J. J. Biol. Chem. 141 : 509. 1941.
15. YOU, R. W. and SELLERS, E. A. Endocrinology, 49 : 374. 1951.

STUDIES ON THE ISOLATION AND NATURE OF THE 'TERREGENS FACTOR'[1]

By M. O. Burton, F. J. Sowden, and A. G. Lochhead

Abstract

A procedure is described for the production and concentration of the 'terregens factor' (TF), a bacterial growth promoting substance synthesized by *Arthrobacter pascens* and essential for the growth of *Arthrobacter terregens*. From culture filtrates of *A. pascens* cultivated in a medium of inorganic salts and sucrose, concentrates of TF may be obtained that are active at 0.001 μgm. per ml., heat stable and contain about 12.7% nitrogen. Acid hydrolysis yielded a number of amino acids, including glutamic acid, glycine, α-alanine, valine, leucine, proline, lysine, and arginine, as well as some unidentified compounds; however, TF does not appear to be a low molecular weight straight chain peptide.

Although TF contains no iron, it combines readily with ferrous or ferric iron to form reddish-brown complexes with this metal. Activity for *A. terregens* is shown by certain iron containing complexes as hemin, coprogen, and ferrichrome. On the other hand none is shown by cytochrome or pulcherrimin; however, aspergillic acid, structurally related to the latter, possesses some growth promoting activity for the test organism.

Introduction

A previously undescribed growth factor for bacteria was reported recently by Lochhead and Burton (6). The factor was first recognized to be present in aqueous extracts of soil which were found to promote the growth of an organism, isolated from soil, that was unable to develop in otherwise adequate media which included yeast extract. The factor could not be replaced by the B vitamins, purines, pyrimidines, or other growth-promoting substances tested, with the exception of hemin, which evoked a partial response of the test organism, and concentrated liver extract.

It was found that the factor could be synthesized by a bacterium, also isolated from soil, having very simple nutritional requirements, and that the culture filtrate of this organism, on partial purification, showed growth promoting activity at low concentrations. The organism requiring the growth factor, as well as that synthesizing it, were previously undescribed species, and were named respectively *Arthrobacter terregens* and *Arthrobacter pascens* (6). The substance required by the former is now referred to as the 'terregens factor' (TF). Following a determination of the more specific nutritional requirements of *A. terregens* (1) it was possible to make use of this organism in developing an assay procedure based on turbidity measurement.

The slight activity shown by hemin in promoting growth of *A. terregens* suggested a possible relationship between the terregens factor and 'coprogen', an iron-containing pigment reported by Hesseltine *et al.* (4) to be active as a growth factor for certain fungi of the genus *Pilobolus* for which hemin provided slight stimulation. A substance possibly related to coprogen was described

[1] *Manuscript received March 26, 1954.*

Joint contribution from the Bacteriology Division (No. 373) and the Chemistry Division (No. 251), Science Service, Canada Department of Agriculture, Ottawa, Canada.

by Neilands (7) who reported the isolation from cells of the fungus *Ustilago sphaerogena* of an iron pigment to which the name 'ferrichrome' was given. The material was stated to be active in promoting the growth of *Pilobolus* sp. The iron content of coprogen and ferrichrome was reported as 6.61% and 7.35% respectively; on the other hand, preliminary tests on unpurified concentrates indicated that TF contained little or no iron. However, samples of coprogen and ferrichrome were found to show activity for *A. terregens*.

Separation and Concentration of the Terregens Factor

For production of TF, 300-ml. quantities of medium contained in 1000-ml. Erlenmeyer flasks are inoculated with *A. pascens*, using 1% of a 48-hr. liquid culture. The medium is prepared by adding to 1 liter distilled water: monohydrogen potassium phosphate, 1.0 gm.; $MgSO_4 \cdot 7H_2O$, 0.2 gm.; calcium chloride, 0.1 gm.; sodium chloride, 0.1 gm.; and $FeCl_3 \cdot 6H_2O$, 0.01 gm. The solution is adjusted to neutrality, heated to boiling, allowed to cool, and filtered, after which 5 gm. sucrose and 1.0 gm. potassium nitrate are added. The medium is then adjusted to pH 6.8 and dispensed in flasks. After addition of the inoculum, the flasks are agitated in a shaking machine at approximately 20°C. for four days.

Following incubation, the culture is adjusted to pH 7.0, partially clarified in a centrifuge, and condensed under vacuum to 1/15 volume. The condensate (cold) is poured with stirring into 10 times its volume of 50 : 50 ethanol–acetone at 0°C. After standing overnight the supernatant is decanted, evaporated to dryness, and taken up in water.

The water fraction, after 3/4 saturation with ammonium sulphate, is extracted with successive half-volumes of benzyl alcohol until the alcohol layer is colorless. The alcohol extracts are combined and after centrifuging are washed with water half-saturated with ammonium sulphate and again clarified. To the benzyl alcohol fraction in a separatory funnel are added three volumes of ether and 1/6 volume of water. The mixture is shaken and the water separated off, the extraction with water being twice repeated. The aqueous extract, after being shaken with 1/3 volume of ether and separated, is dried under vacuum and extracted with warm methanol. A white, nonactive precipitate is removed by centrifugation. The active solution can then be brought to dryness and taken up in water. By this procedure highly active concentrates have been obtained. By means of paper chromatography with 50% ethanol as solvent and 0.5% ferric chloride as developer, a fairly sharp band was obtained (R_f approx. 0.85) at which the activity was largely concentrated.

For the assay, a medium of the following composition, per liter, is used:– monohydrogen potassium phosphate, 1.0 gm.; $MgSO_4 \cdot 7H_2O$, 0.2 gm.; calcium chloride, 0.1 gm.; sodium chloride, 0.1 gm.; $FeCl_3 \cdot 6H_2O$, 0.01 gm.; glucose, 1.0 gm.; casamino acids (Difco), 1.0 gm.; thiamine, 800 μgm.; calcium pantothenate, 800 μgm.; and biotin, 2 μgm.

The inorganic salts, with the exception of ferric chloride, are dissolved in approximately 3/4 of the final volume of distilled water, adjusted to neutrality, heated to boiling, cooled, and filtered. Glucose, casamino acids, and the vitamins are added and the volume brought up to four-fifths of the final amount. The medium, after being adjusted to pH 6.8, is dispensed in 20 ml. portions to which are added respectively solutions of the test material at different levels and sufficient distilled water to bring the volume to 25 ml. Duplicate 10-ml. portions are added to 50-ml. Erlenmeyer flasks to which, after sterilization, the iron salt is added aseptically. The contents of the flasks are inoculated with one drop of a suspension of *A. terregens* prepared by centrifuging 10 ml. of a four to five day culture in the above assay medium containing TF, washing the cells three times in sterile saline, and making the volume up to 10 ml. Cultures are incubated in a reciprocating shaker (80 strokes per minute; horizontal traverse of 2.75 in.) for 48 hr. at 20°C. after which turbidity measurements are made in a spectrophotometer, allowing for uninoculated controls.

Tests of the activity of a typical concentrate are shown in Fig. 1. It is observed that *A. terregens* commences to show response to the factor at a concentration of 0.001 μgm. per ml. under the conditions of assay described above. TF, tested at concentrations as high as 1–150 by means of the disk assay procedure, showed no antibiotic activity against *Micrococcus pyogenes* var. *aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, or *Bacillus cereus*.

Chemical Properties of the Terregens Factor

The factor could not be distilled by short path distillation at 120°C. and 0.001 mm. Hg pressure. It was not destroyed by heating at 150°C. for two hours, but was almost completely inactivated by heating to 200°C. for that period. It could not be adsorbed on anion exchange resins, but was strongly

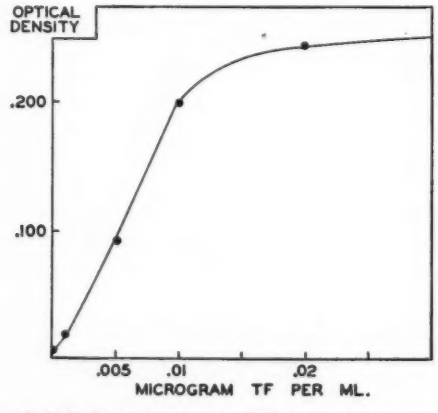


FIG. 1. Response of *Arthrobacter terregens* to TF concentrates prepared from culture filtrates of *Arthrobacter pascens*.

adsorbed on cation resins and on charcoal; it could be incompletely removed from these two latter adsorbents by ammoniacal solutions.

Analysis of the concentrate showed that it contained only traces of iron. Nitrogen determinations made on three different preparations of the concentrate gave percentages of 12.2, 12.7, and 13.4 respectively; the difficulty of securing accurate moisture-free weights on small samples of the amorphous material may account for some of the variability of the nitrogen data.

Samples of different concentrates were hydrolyzed in sealed tubes with 6 *N* hydrochloric acid at 105°C. for 20 to 24 hr., the tubes opened, and the hydrolyzates evaporated to dryness under vacuum and wetted and dried several times to remove traces of hydrochloric acid. The hydrolyzates were then chromatogrammed in 80% phenol and butanol – acetic acid – water (4:1:1). The phenol chromatograms indicated the presence of glutamic acid, glycine, α-alanine, and a large amount of material that ran near the solvent front. Two-dimensional chromatograms in 80% phenol and butanol – acetic acid – water also indicated the presence of the above-mentioned acids and separated the material that ran near the solvent front in phenol into seven or eight distinct bands. When the material was chromatogrammed in butanol – acetic acid – water 11 or 12 bands were formed. The results suggested the presence of glutamic acid, glycine, α-alanine, valine, leucine, proline (confirmed with the isatin test), lysine, and arginine. No evidence for the presence of any sulphur-containing amino acid was noted when a butanol chromatogram was sprayed with the iodine – sodium azide reagent. There were no indications of the presence of sugars, purines, or pyrimidines in the hydrolyzates. The chemical nature of coprogen has not been disclosed but ferrichrome has been reported to contain glycine and a diamino acid (8).

An attempt was made to degrade the material by the action of trypsin, but paper chromatography of the hydrolyzate of the material resulting from trypsin digestion indicated that trypsin had had little effect on the concentrate; there was no increase in the biological activity of the factor after trypsin hydrolysis.

Determinations were made of the free amino groups at the end of the peptide chain using Sanger's (10) dinitrofluorobenzene procedure and a modification (9) of Edman's (2) phenylisothiocyanate method on two different preparations of TF. The latter indicated that some valine and traces of one or two other amino acids were combined in such a manner that their amino groups were free. Traces of DNP–valine and the DNP derivative of one other amino acid were found by the use of Sanger's method. In all instances larger amounts of amino acid derivatives should have been recovered if the TF were a simple straight chain peptide with a relatively low molecular weight. Somewhat larger amounts of valine were found by Edman's method than by Sanger's.

Although TF, as isolated, contains little or no iron it combines readily with ferrous or ferric iron to form complexes that are reddish-brown in color.

Such color is characteristic of hydroxamic acids and is similar to that given by some proteins and other nitrogen containing compounds (3, 5, 11). The TF–iron complex was purified by extraction into benzyl alcohol then, on treatment with ether, into water. This purification was repeated and the complex was found to contain 7.6% iron. To test the effect of concentrations of ferrous or ferric iron on complex formation, aqueous solutions of the concentrate (5 mgm. per ml.) were treated with equal and with 1/5 volumes of 0.1 *M* solutions of ferrous and ferric iron. The complex formed was extracted into benzyl alcohol and, on treatment with ether, into water, the water evaporated, and the complex dissolved in methanol. The ferric iron complexes were only slightly soluble in methanol and recoveries were low (about 15–20%); the ferrous complexes were much more soluble in methanol and about four-fifths of the original material was recovered in the methanol solution. The percentages of iron in the complexes after drying from the methanol solutions were as follows: 5.5% and 4.2% when treated with equal and 1/5 volumes of 0.1 *M* ferrous solution respectively; and 2.8% and 3.4% when treated with equal and 1/5 volumes of 0.1 *M* ferric solution. The indications are, therefore, that TF does not form a stable stoichiometric complex with iron; however, the pH was not controlled and the treatment and purification of the complex were carried out in the presence of air.

Crystallization of TF has not been accomplished, the purest concentrates so far obtained maintaining an amorphous form. However, from solutions of the ferrous, though not the ferric, complex there may be observed, on evaporation, abundant formation of needle-shaped crystals resulting from friction applied to the amorphous residue. Their separation from solution has not been achieved. Ultraviolet absorption spectrum tests with TF and the TF-ferric complex showed a regular curve of receding values through the range 300–500 mμ. On the other hand the ferrous complex showed a rise at 360 mμ to a maximum at 450 mμ (Fig. 2).

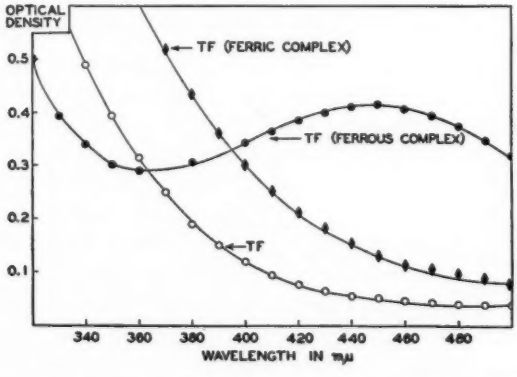


FIG. 2. Absorption spectra of TF and of ferric and ferrous complexes.

Activity of Certain Iron-containing and Related Compounds

It was previously reported (6) that hemin, at concentrations between 0.1 and 0.5μgm. per ml., showed some activity in promoting growth of *A. terregens*. Consequently, further tests were made on a number of other iron-containing compounds, including cytochrome c, coprogen (4), ferrichrome (7), and pulcherrimin, the last-named substance having been recently isolated by Kluyver *et al.* (5) as the compound responsible for the pigmentation of *Candida pulcherrima*. Since these authors noted a configurational relationship between pulcherrimin and aspergillic acid, the latter compound was also tested. As noted in Table I, coprogen and ferrichrome showed high activity for *A. terregens* analogous to that provided by TF while aspergillic acid was active at considerably lower dilutions, no growth promoting effect being noted at concentrations below 1 μgm. per ml. Cytochrome c and pulcherrimin were found to be inactive at the concentrations tested. The relationship of TF to the other substances showing growth promoting effect is being made the subject of further study.

Acknowledgments

The authors are indebted to Dr. H. Katznelson for valuable advice and for having made the absorption spectrum tests, to Mr. R. Levick for the iron analyses, to Dr. A. R. Whitehill, Dr. J. B. Neilands, Prof. A. J. Kluyver, and Dr. J. B. Dutcher for having kindly furnished, respectively, samples of coprogen, ferrichrome, pulcherrimin, and aspergillic acid, and to Miss Winnifred Ryan for very effective technical assistance.

TABLE I

EFFECT OF VARIOUS IRON-CONTAINING AND IRON-COMPLEXING SUBSTANCES IN PROMOTING GROWTH OF *A. terregens*

Substance	Concentration (μgm./ml.)				
	10	1	0.1	0.01	0.001
Hemin	0	0	+	0	0
Cytochrome*	−	0	0	0	0
Coprogen**	+	+	+	+	−
Ferrichrome	+	+	+	+	+
Pulcherrimin	0	0	0	0	0
Aspergillic acid	+	+	0	0	0
TF	+	+	+	+	+

*Not tested at concentrations higher than 1 μgm./ml.
**Not tested at concentrations lower than 0.01 μgm./ml.

References

1. BURTON, M. O. and LOCHHEAD, A. G. Can. J. Botany, 31 : 145. 1953.
2. EDMAN, P. Acta Chem. Scand. 4 : 283. 1950.
3. FIALA, S. and BURK, D. Arch. Biochem. and Biophys. 20 : 172. 1949.
4. HESSELTINE, C. W., PIDACKS, C., WHITEHILL, A. R., BOHONOS, N., HUTCHINGS, B. L., and WILLIAMS, J. H. J. Amer. Chem. Soc. 74 : 1362. 1952.
5. KLUYVER, A. J., VAN DER WALT, J. P., and VAN DER TRIET, A. J. Proc. Natl. Acad. Sci. U.S. 39 : 583. 1953.
6. LOCHHEAD, A. G. and BURTON, M. O. Can. J. Botany, 31 : 7. 1953.
7. NEILANDS, J. B. J. Am. Chem. Soc. 74 : 4846. 1952.
8. NEILANDS, J. B. Abstr. Am. Chem. Soc. 1953. Div. Biol. Chem. p. 31C.
9. OTTESEN, M. and WOLLENBERGER, A. Nature, 170 : 801. 1952.
10. SANGER, F. Biochem. J. 39 : 507. 1945.
11. SHADE, A. L., REINHART, R W., and LEVY, H. Arch. Biochem. and Biophys. 20 : 170 1949.

STUDIES ON ACCLIMATIZATION AND ON THE EFFECT OF ASCORBIC ACID IN MEN EXPOSED TO COLD[1]

By J. LeBlanc, M. Stewart, G. Marier, and M. G. Whillans

Abstract

This experiment was planned to study the problem of acclimatization in humans and to determine the effect of ascorbic acid in men exposed to cold while being fed a normal or survival ration. Ascorbic acid has greatly improved the resistance of men exposed to cold and fed a survival ration. No beneficial effect was observed when the subjects were fed a normal ration. This difference in response may be due to the fact that the experimental conditions differed somewhat between these two experiments. In any event, the subjects on a restricted food intake were certainly under greater conditions of stress. Evidence of acclimatization was obtained with survival rations but not with normal rations. Some conclusions have been made on the use, by men exposed to cold, of survival rations composed exclusively of carbohydrates. Finally, it is estimated that 2800 calories is the daily requirement for men relatively inactive, wearing only shorts, low shoes, and socks, and exposed to an ambient temperature of 60°F.

Introduction

Many physiological changes have been described in men exposed to cold. Specific reactions (diuresis, change in blood volume, increased caloric output, etc.), as well as nonspecific ones (enhanced adrenocortical activity), are known to occur at low environmental temperatures. Some investigators questioned the importance of these changes in improving resistance to cold (1). In other words, no real evidence has been given showing whether or not acclimatization to cold exists in human beings. This experiment was partly designed to clarify some aspects of this question. We have studied certain specific manifestations of cold (drop in rectal and skin temperatures, subjective sensation of discomfort, diuresis, etc.) to determine whether there was any time sequence modifications of these responses. We felt that a return of these variations towards normal would indicate signs of increased resistance or acclimatization to cold.

The effect of large doses of ascorbic acid on resistance to cold was also investigated in this experiment. Some investigators (27, 30, 5, 4, 2) have shown an increased resistance to different stresses, attributable to ascorbic acid, and Dugal and co-workers demonstrated the beneficial effect of this vitamin in resistance to cold. It was shown that ascorbic acid increases the resistance of guinea pigs to cold (7) and prevents, to a certain extent, some of the manifestations of cold in rats (8, 29). These same workers have shown that pre-exposure of monkeys to a mild cold does not enhance resistance to intense cold—as measured by the fall in rectal and muscular temperatures—unless the animals pre-exposed to cold receive at the same time large doses of ascorbic acid (9). These results were partially explained by a hyperactivity of the adrenal cortex (29). Furthermore, evidence was given showing the synergetical action of ascorbic acid and ACTH (3, 10, 11).

[1] *Manuscript received December 22, 1953.*
Contribution from Defence Research Medical Laboratories, Toronto, Canada.

In view of these results, larger quantities of ascorbic acid have been added to certain rations used in the Armed Forces. No evidence has ever been given, however, showing that these supplements increase the resistance of men to low environmental temperatures. On the contrary, Glickman *et al.* have failed to demonstrate any beneficial effect of large doses of vitamin C on the tolerance to cold (16). In view of this conflicting evidence between laboratory animals and men, an experiment was planned to clarify this question. In the first part of this work the subjects were fed a survival ration, whereas a normal ration was used in the second part.

Part I. Effect of Ascorbic Acid on Men Exposed to Cold and Fed a Survival Ration

Description of the Experiment

Experimental Plan

Twelve airmen were confined to a room for 13 days. Six of these subjects were receiving 525 mgm. of ascorbic acid per day, the others 25 mgm. None, however, knew to which group he belonged, since all subjects received four pills a day. In the low vitamin C group, one pill contained 25 mgm. of ascorbic acid, the other three were placebo. After a control period of two days, when the room was kept at 79 ± 1°F., the temperature was maintained at 59 ± 1°F. for the last 11 days. On the basis of Clo values, it could be mentioned that, to be comfortable at this temperature, approximately 2.7 Clo are needed (14). The subjects were given normal camp ration during the control period, fasted for the first day of the experimental period, and were kept on a low calorie diet for the last 10 days. The low calorie rations, composed exclusively of carbohydrates in the form of jelly candy, supplied 550 calories per day. During both periods the subjects wore shorts, socks, and low shoes during the day and reclined quietly. From 8.30 p.m. to 8.30 a.m. they covered themselves with one sheet and one wool blanket. The daily water intake was measured, but unrestricted.

Tests and Methods

At 8.30 a.m. the basal metabolic rate was taken, and the subjects were weighed after they had urinated.

At 10.00 a.m. and 1.00 p.m. a drop of blood was taken for a direct count of red and white blood cells, as well as eosinophiles (17).

At 10.15 a.m. the rectal and skin temperatures, pulse, and blood pressure were recorded. The same measurements were made at 1.15 and 4.15 p.m.

The rectal temperature was obtained by clinical thermometers inserted to a distance of 40 mm. The skin temperature measurements were obtained with thermocouples made of 36 gauge copper and constantan wires. The thermocouples were encased in Y-shaped glass tubing, which allowed an even pressure of the thermocouples' junction with the skin (15). Readings were made on 15 parts of the body in order to compute the average skin temperature. The temperature of the feet, however, was not observed since our subjects were wearing woollen socks and shoes.

The 24-hr. urine specimens were preserved with oxalic acid. Determinations of ascorbic acid by the indophenol (12) and phenylhydrazine methods (24), chlorides (18), ketone bodies (31), nitrogen (13), and 17-ketosteroids (6) were made on each daily urine sample.

On the 4th and 8th day in the cold, whole blood vitamin C concentrations were measured. We also looked into the possibilities of getting subjective evidence by asking our subjects their personal estimations of the cold severity on the 2nd and 10th day, and of the beneficial effect of vitamin C on the 11th day.

Results

A. Metabolic Exchanges

(1) Fig. 1 shows that there was a very sharp drop in weight in the first five days, amounting to as much as 5 lb. on the first day. From the 6th to the 13th day, the weight loss is smaller and remains constant (average of 0.73 lb. per day). The difference in the water output between the first and the last part of the cold period explains the greater weight loss observed during the first days of exposure to cold. Confirming this interpretation is the fact that the tissue catabolism, as will be shown subsequently, is approximately the same during the 11 days of the cold exposure period.

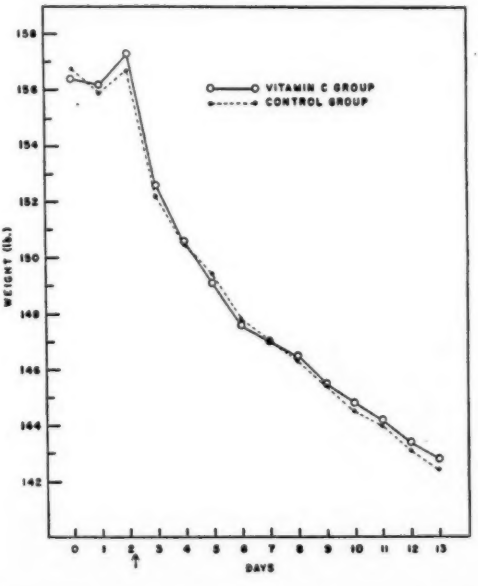


FIG. 1. Effect of high (525 mgm.) and low (25 mgm.) vitamin C supplements on the body weight of men exposed to cold (60°F.) and fed a survival ration (550 cal./day).

(2) The total nitrogen excretion, as illustrated in Fig. 2, drops on the first day of exposure to cold and remains low until the end of the experiment. However, since our subjects were completely deprived of external sources of proteins, the nitrogen, found in the urine, must come from a destruction of tissue proteins, which amounts to about 50 gm. per day.

(3) Fig. 2 shows that there is in both groups a significant increase of ketone bodies in the urine. As was noticed for the nitrogen excretion, the changes observed in the first days of the cold period remain the same throughout the experiment.

(4) During the last seven days of the cold period, the basal metabolic rates of the subjects receiving 525 mgm. of vitamin C are significantly lower than those of the other group. Fig. 3 shows this difference.

B. Water Balance

(1) The unrestricted water intake, as shown in Fig. 4, is very low at all times and is an indication of a physiological adjustment to restricted calorie intake in a cold environment. The gradual increase corresponds to a decrease in the cold diuresis.

(2) Fig. 4 shows a very large water excretion in the first day of exposure to cold, although the intake is very low. This negative water balance lasts

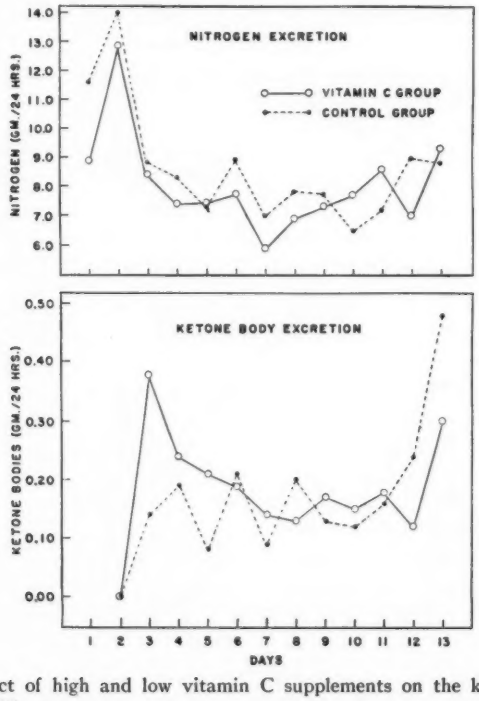


FIG. 2. Effect of high and low vitamin C supplements on the ketone bodies and nitrogen excretion.

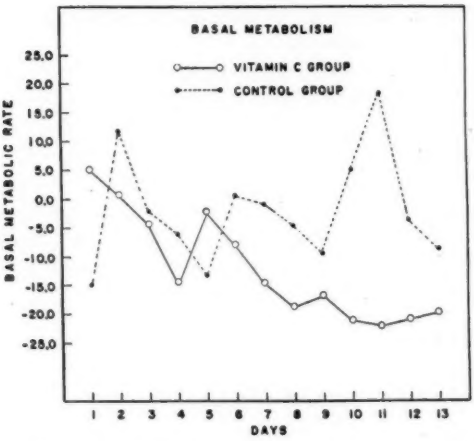


FIG. 3. Basal metabolic rate in the high and low vitamin C group.

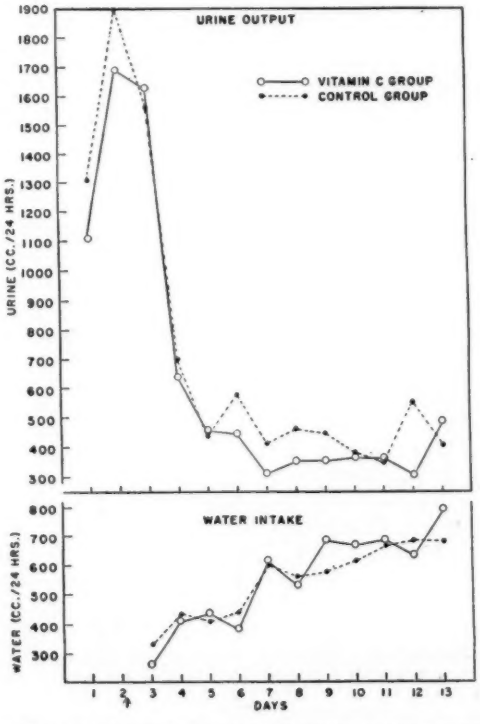


FIG. 4. Variations of urine output and water intake in the two groups studied.

for a few more days. During the rest of the experiment the water intake is larger than the water output.

(3) The very sharp drop in chlorides, which remains constant at very low levels after four or five days in the cold, resembles that which was observed for water excretion. However, the chloride excretion as shown in Table I drops to about three per cent of what it was in the first day of cold, whereas the corresponding value for the urine excretion is 25%. Blood chlorides determinations would tell us whether this decrease is due to a change in the threshold for chlorides excretion or is due to the fact that our subjects, being on almost complete salt restriction, the only source being the drinking water, had very low blood chlorides levels.

C. Blood Analysis

(1) An initial increase in the erythrocyte counts is reported in Fig. 5. This increase is followed by a return towards normal values during the last days of the cold exposure.

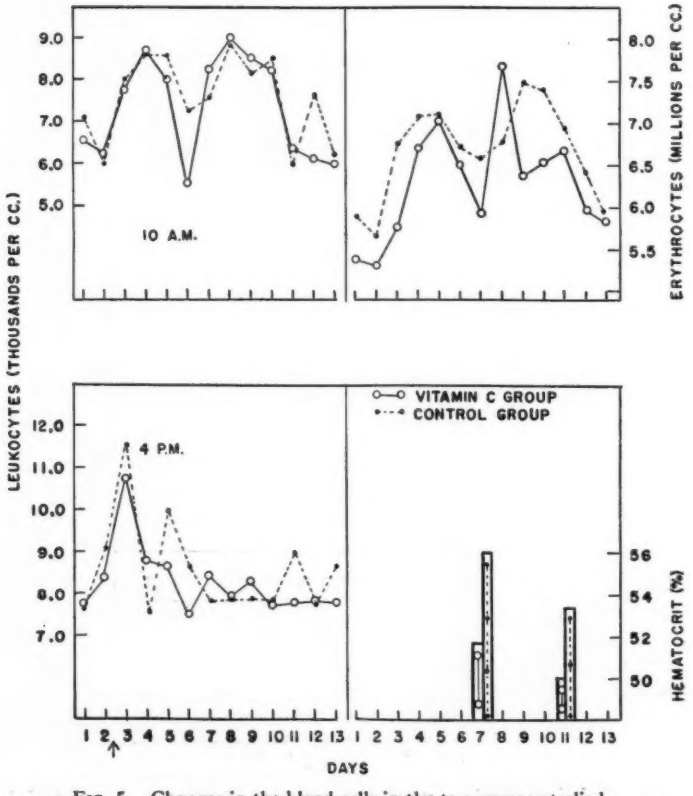


FIG. 5. Changes in the blood cells in the two groups studied.

TABLE I

DAILY VARIATION OF TOTAL CHLORIDE EXCRETION (GM. PER DAY) IN GROUP 1 (RECEIVING 525 MGM. VITAMIN C PER DAY) AND GROUP 2 (25 MGM. PER DAY)

Days	1	2	3	4	5	6	7	8	9	10	11	12	13
Group 1	12.38	18.49	14.31	3.32	1.89	0.86	0.55	0.29	0.23	0.20	0.24	0.28	0.34
Group 2	13.47	19.31	16.52	3.17	1.01	1.09	0.43	0.40	0.32	0.20	0.15	0.27	0.11

TABLE II

MORNING, NOON, AND AFTERNOON VARIATIONS OF RECTAL TEMPERATURE (°F.) IN GROUP 1 (RECEIVING 525 MGM. OF VITAMIN C PER DAY) AND GROUP 2 (25 MGM. PER DAY)

	Days	1	2	3	4	5	6	7	8	9	10	11	12	13
a.m.	Group 1	98.6	98.7	97.9	98.5	98.3	98.4	98.8	98.6	98.7	98.7	98.5	98.6	98.4
	Group 2	98.8	98.7	98.3	98.6	98.9	98.4	98.9	98.8	98.9	98.6	98.6	98.5	98.3
Noon	Group 1	98.4	98.8	97.9	98.1	97.9	98.1	98.7	98.4	98.5	98.3	98.4	98.5	98.2
	Group 2	98.5	98.7	97.7	97.9	98.4	98.4	98.7	98.2	98.3	98.6	98.3	98.5	98.3
p.m.	Group 1	98.9	98.7	97.9	97.9	98.2	98.2	98.6	98.2	98.4	98.1	98.1	98.3	98.1
	Group 2	98.9	99.2	97.8	98.3	98.5	98.2	98.6	98.0	98.5	98.2	98.8	98.4	98.2

(2*a*) The values obtained for leucocyte counts in the morning are from the same blood samples as those used for the red blood cells counts. The results, as shown in Fig. 5, parallel those obtained for erythrocytes. Indeed, the increase observed at first is followed by a drop to normal values.

(2*b*) The results for afternoon counts are similar to those obtained in the morning with the difference, however, that the return to normal value occurs five or six days earlier in this case. This is illustrated in Fig. 5.

(3) Fig. 5 also shows a decrease in the hematocrits at the end of the cold exposure period. The difference between the two groups is not statistically significant.

The results reported so far indicate no statistically significant difference between the groups studied, except in the case of metabolic rate where it was lower in the high vitamin C group.

D. Body Temperature

(1) The results from skin temperature measurements are reported in Fig. 6.

(1*a*) After about five days in the cold, the skin temperature values of both groups are higher than at the time of initial exposure to cold and are higher in the high than in the low vitamin C group.

(1*b*) Although on the first day the skin temperature is lower in the afternoon, values for this time of the day have reached the same levels at the end of the experiment as those observed in the morning or midday.

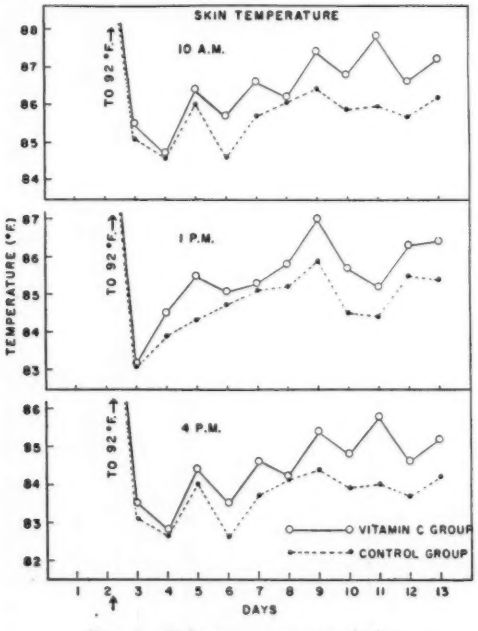


FIG. 6. Skin temperature variations.

(2) There is no difference in the rectal temperature readings between the groups studied. The values in the morning are approximately the same in the control and cold periods. There is an initial fall for midday readings, which lasts for about five days in the cold, where the values reach a normal level. Afternoon readings are lower than normal at all times in the cold. These results are reported in Table II.

E. Adrenal Activity

(1) Because of the daily and individual variations in the 17-ketosteroids excretion, the slight difference between the two groups is not statistically significant. There is, however, a very large diminution in both groups which lasts till the end of the experiment, as shown in Fig. 7.

(2) The most obvious difference in the eosinophile counts is the one observed between the groups, morning and afternoon values being significantly higher (at the 5% level) in the high vitamin C group. It should also be pointed out that in both groups there is an increase in the morning and a decrease in the afternoon. These results are illustrated in Fig. 8.

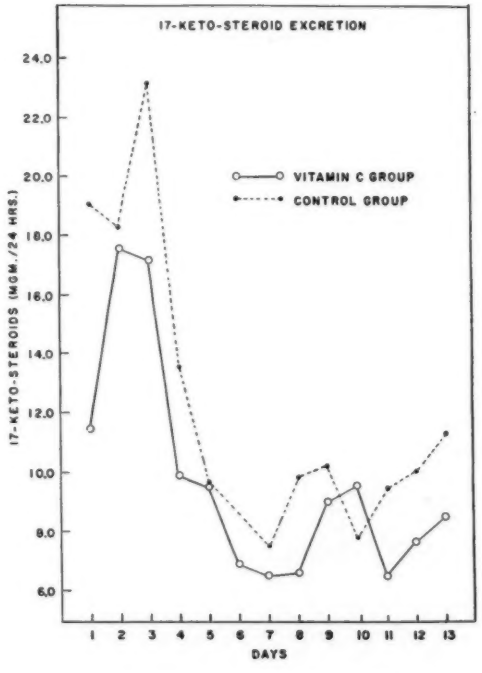


FIG. 7. Changes in the 17-ketosteroid excretion during the experimental period.

F. Foot Trouble

On coming out of the experimental room, all subjects experienced more or less trouble with their feet. A description of the foot trouble observed is reported in Table III. It is obvious from these results that the foot troubles were more severe and lasted longer in the low vitamin C group.

G. Subjective Evidence

(1) The subjects were asked at times to rate the severity of the three stresses they were exposed to (cold, hunger, and confinement). On the second day of the experimental period they all rated the cold first, hunger second, and confinement third as a source of discomfort. On the 10th day, however, they estimated hunger as being the worst, cold the second, and confinement last, as before.

(2) Two days before coming out of the cold room our subjects, when asked which group they were in on the basis of comfort and alertness, gave the following answers: Of the six men in the high vitamin C group, five thought they received more vitamin C and one less vitamin C. In the other group, four said they had low vitamin C pills, one high vitamin C, and one was undecided.

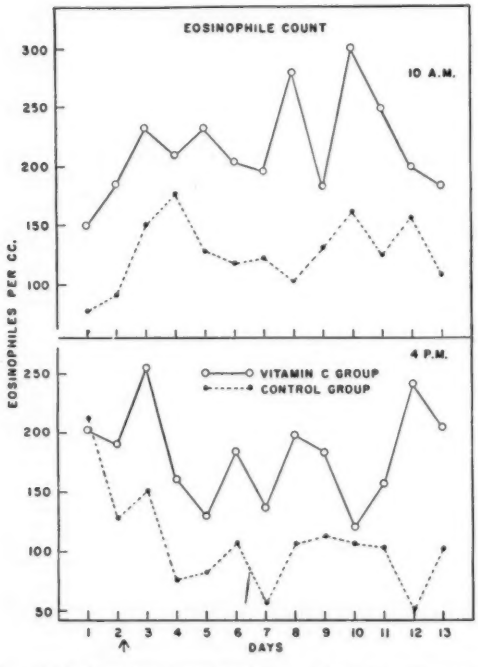


FIG. 8. Variations in the eosinophiles of the two groups studied.

TABLE III

DESCRIPTION OF FOOT TROUBLE

Subject		Duration, days
	Vitamin C intake 525 mgm. per day	
J.B.	Some muscular weakness	2
F.J.	Slight burning sensation	2–3
A.G.	Sensation of pins and needles	2
B.C.	" " " " "	2
W.B.	" " " " "	1
R.J.	Soreness of the soles with some pain when walking	7
	Vitamin C intake 25 mgm. per day	
M.B.	Severe aching, which would occasionally keep him awake at night with extreme difficulty in walking	15
R.W.	Noticeable swelling of feet with difficulty in walking	10
S.P.	Swelling of feet with difficulty in walking	10
L.W.	Some muscular weakness with occasional pain	8
A.G.	Frequent pains and soreness	10
G.H.	Sensation of pins and needles	3

H. Vitamin C Determinations

In the high vitamin C group, the total vitamin C excretion in the cold is about 475 mgm. per day, and the concentration in the blood is approximately 1.6 mgm.%. The corresponding values for the other group are 5.5 mgm. and 0.8 mgm.% respectively. These results are illustrated in Fig. 9.

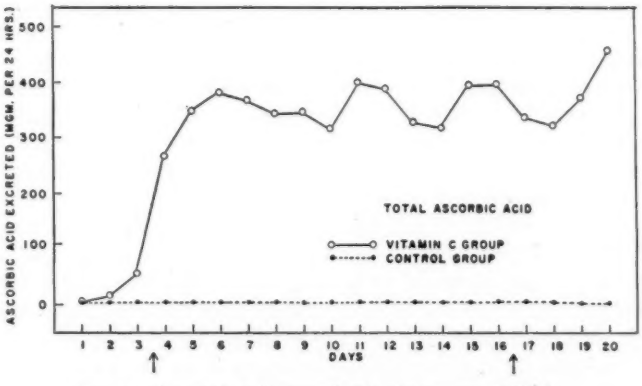


FIG. 9. Ascorbic acid excretion in the groups studied.

Discussion

Since we have three main factors involved in this experiment, we feel that an analysis of the results should be made under three general titles:

I. Ascorbic acid.

II. Cold acclimatization.

III. Survival rations.

I. Ascorbic Acid

When comparing a group of men receiving 25 mgm. of ascorbic acid with another getting 525 mgm. of the same vitamin, we have noticed an increased resistance to cold in the high vitamin C group. One of the most striking differences between the two groups is the higher skin temperature observed in the high vitamin C group. On the cheek, torso-front, and upper arm the skin temperature is respectively 1.0, 2.0, 1.5°F. higher in the high than in the low vitamin C group, whereas on forehead, lower arm, hand, and finger it is 0, 0.5, 0, 0°F. It seems, then, that the higher skin temperatures observed in the high vitamin C group correspond to locations where there is more subcutaneous fat. This beneficial effect is not detrimental to the rest of the body, since the rectal temperature is the same in both groups. (This difference between the two groups might be due to a vasodilatation.) By referring to the skin temperature readings for both groups, it seems as if the high vitamin C group is exposed to a less severe cold than the low vitamin C group. This assertion is confirmed by the subjective evidence. Another result that we consider of great practical significance is the difference in foot troubles observed between the two groups. Indeed, the addition of 525 mgm. of ascorbic acid to the diet decreased enormously the intensity and the duration of the symptoms noticed in the low vitamin C group. No definite answer can be given at the moment as to whether this foot trouble is caused by cold and/or survival rations. In the same manner, an explanation for the beneficial effect of vitamin C cannot, in this case, be given as yet. It may be said however, that the enhanced peripheral circulation induced by vitamin C, as indicated by the higher skin temperature, may prove to be an important factor in the reduction of foot trouble of the same nature as that noticed in this experiment. In any event, this effect of vitamin C acquires great importance with regard to military personnel exposed to cold and reduced rations simultaneously. We have mentioned a very low 17-ketosteroids excretion and no difference between the groups studied. On the other hand, it has been shown on laboratory animals exposed to cold, that large doses of vitamin C induced hyperactivity of the adrenal cortex (29). This contradiction is only apparent when it is remembered that the subjects in this experiment were fed survival rations, completely depleted of proteins. It has been proved that, in rats deprived of proteins, the activity of the adrenals is greatly reduced (21). Further experiments have shown that a lack of secretion of ACTH is the cause for this hyposecretion of the adrenal cortex (25).

The lower BMR in the high vitamin C seems in contradiction with the higher skin temperature, noticed in this group. It should be mentioned that the higher skin temperature observed in the high vitamin C group was observed during the day, that is, when the subjects were exposed to cold. The BMR, however, was taken in bed, where the subjects were comfortable and not exposed to cold.

II. Cold Acclimatization

The question of acclimatization, as we have mentioned in the introduction, has intrigued many investigators and left many of them perplexed. This experiment has furnished results which clarify some aspects of the problem. After the subjects have been about five days in the cold, the diuresis is greatly reduced, the skin temperature is increased by 2° to 3°F., and the leucocytes (afternoon determination) are back to normal. The morning determinations of the erythrocytes and leucocytes also return to normal levels, although the process is slower. By that time, about five days, all these variations have reached a certain level or equilibrium, which lasts till the end of the experiment. Along with these observations, it was found that the subjects felt warmer during the last days of the experiment. Confirming this result, we had noticed that the subjects were obviously shivering more during the first part of the experiment. One may suppose that they had acquired the facility of shivering more economically. We have, then, observed a definite acclimatization of men exposed to cold, which diminished the discomfort encountered at low environmental temperatures.

III. Survival Rations

As expected, the calories given to our subjects were not sufficient, as evidenced by a body protein and fat catabolism, which is reflected in the weight loss. This experiment furnishes useful information as to the amount of water needed by men exposed to cold in a state of semistarvation. Under these conditions, and on a basis of voluntary intake, the water intake during the first few days, as shown by our results, is about 400 cc. Once the subjects are acclimatized, the water intake has increased and is between 600 and 700 cc. These rations, composed exclusively of carbohydrates, do not prevent a certain degree of acclimatization, as we have observed. It should be noticed however, that lack of proteins has greatly reduced the activity of the adrenals. The low osmotic pressure of the blood may have been a concurring factor in causing the foot trouble mentioned. These two last observations need further investigation to determine the possible detrimental effect of carbohydrate survival rations in resistance of men exposed to cold.

Part II. Effect of Ascorbic Acid on Men Exposed to Cold and Fed a Normal Ration

Two series of experiments were performed. In each series, two groups of three soldiers were used; one group receiving 525 mgm. of ascorbic acid and the other 25 mgm.

In *series A*, the six subjects were confined successively for three, 13 and four days in a room where the temperature was maintained at 79 ± 1°, 60 ± 1°, and 79 ± 1°F. respectively. The daily procedure was the same as in the first part of this experiment, except for an additional measurement of oxygen consumption at 4 p.m.

In *series B*, the room was maintained at 79 ± 1°F. for three days. Owing to technical difficulties, the cold room exposure was different from series A. The room was kept at 59 ± 1°F. for eight days. However, because of the impossibility to maintain this temperature for the last five days of the cold period, the subjects were dressed in long wool underwear, wool socks, khaki shirt, battle dress, winter cap, and mitts, and transferred to another room where the temperature was approximately 5°F. They were exposed to this temperature from 9.30 to 12.00 in the morning and from 1.30 to 4.00 in the afternoon. The rest of the day they stayed, with the same clothing as the subjects of series A, in the room at 69°F. The cold exposure period was followed by a two day period when the temperature was 69 ± 1°F. The daily procedure was the same as in series A, except that the skin and rectal temperatures, pulse, and blood pressure in the second part of the cold period were taken at 9.30 a.m., 12.00 noon, 1.30 and 4.00 p.m., instead of 10.00 a.m., 1.00, and 4.00 p.m. as in series A.

In both series, the subjects were fed the Canadian Five-Man Ration Pack.

Results

A. Metabolic Exchanges

(1) There is no important change in the body weight during the initial control period. However, after four days in the cold, the subjects all start to increase in weight, and those in series A gained, throughout the experiment, about 2.5 lb.; those in series B approximately 2.0 lb. There is no difference between the groups. The average daily food consumption is equivalent to 3400 calories.

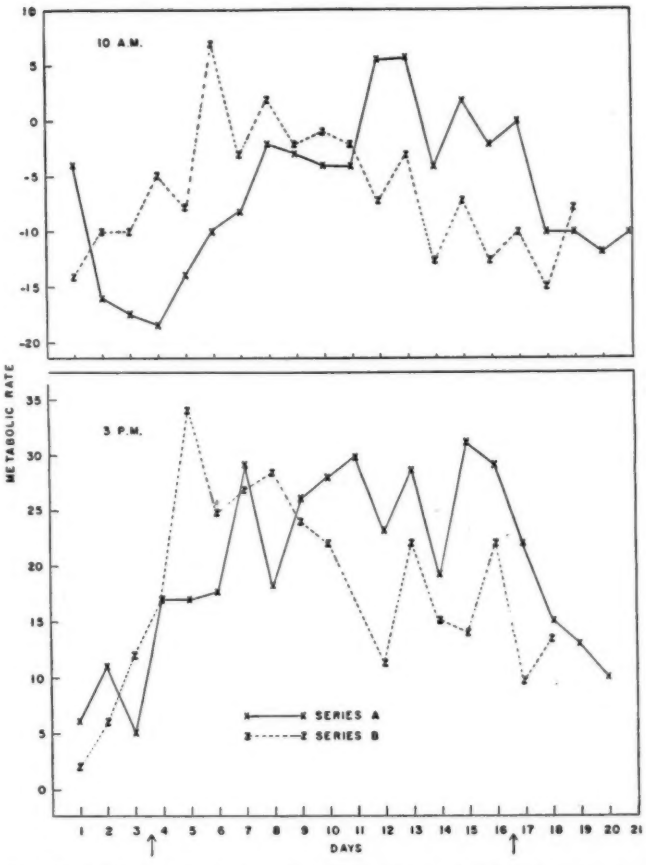
(2) The oxygen consumption, as shown in Fig. 10, increases in both series during the cold period. Values for the afternoon determinations are, however, at higher levels than those made in the morning. There is no difference between the series or groups.

B. Water Balance

(1) The quantities of liquid consumed are the same in both groups and series, and amount to approximately 2700 cc. per day.

(2) There is no significant difference between the urine volume of the two series.

(3) *Electrolytes.*—The amount of chlorides, sodium, and potassium excreted daily or in 100 cc. of urine is the same in both groups and series. There is no change in the daily excretion of these electrolytes between the control and cold period. However, because of the larger quantity of urine excreted in



IG. 10. Metabolic variations of subjects exposed to cold and fed a normal ration.

the cold period, the concentrations of these electrolytes in the urine is smaller during this period. The results for urine and sodium excretion are reported in Fig. 11.

C. Blood Analysis

Determinations of the hematocrit, white and red blood cells have shown no difference between the groups and series.

D. Body Temperature

The only differences in the skin temperature readings are those observed at 10.00 a.m. and 1.00 p.m. when readings are higher in series B than in series A. This difference is explained by the changes in the protocol mentioned previously. The rectal temperature determinations show no significant difference.

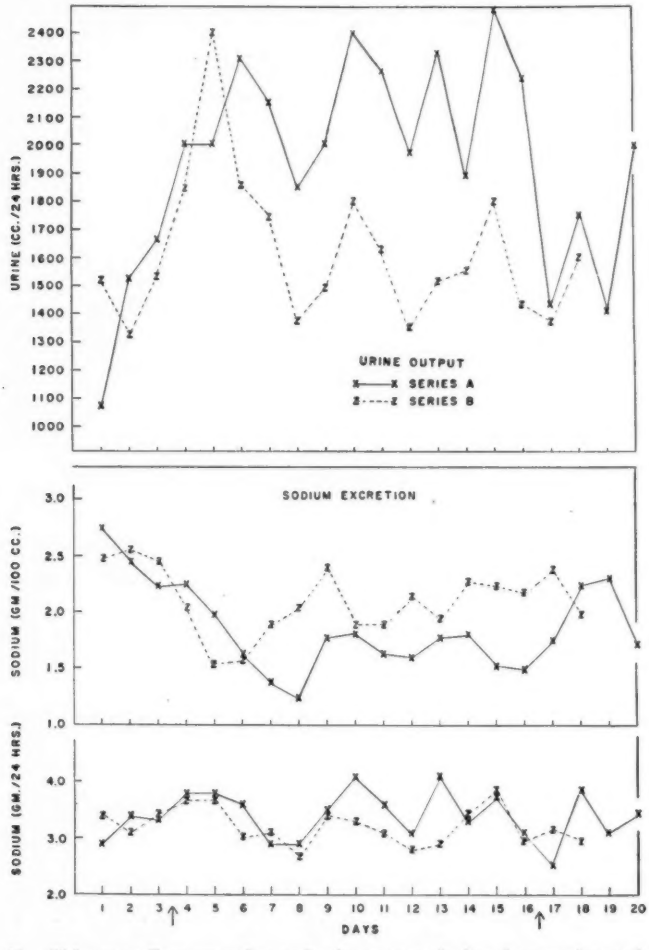


FIG. 11. Urinary sodium excretion and urine output during the experimental periods.

E. Adrenal Activity

(1) There is no difference between the groups or series. But in both series there is, after an initial increase, a drop in the 17-ketosteroids excretion. This is followed by a return to normal values at the end of the cold exposure period, as illustrated in Fig. 12.

(2) There is no difference in the eosinophile counts between the groups or series.

F. Subjective Evidence

Analysis of the results show no significant difference.

G. Vitamin C Determinations

In the high vitamin C group the daily urinary excretion of this vitamin, as shown in Fig. 13, is 375 mgm., and the blood concentration is about 1.2 mgm.%. The corresponding values for the other group are 5.5 mgm. and 0.4 mgm.% respectively.

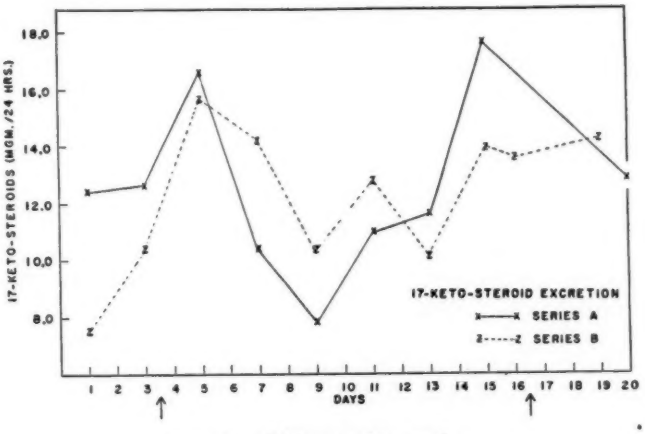


FIG. 12. 17-Ketosteroid excretion.

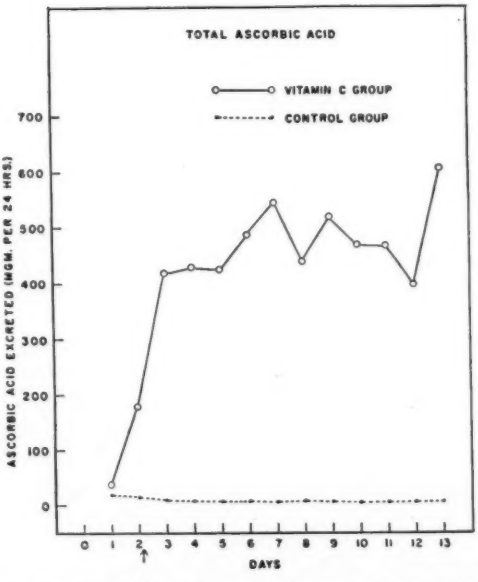


FIG. 13. Total ascorbic acid excretion in the two groups studied.

Discussion

The conditions of stress were certainly greater in the first part of this experiment where survival rations were used. It may well be that with normal rations, the detrimental effects of cold were not sufficient to make obvious the beneficial effects of vitamin C.

There is a difference in the response to cold between all the subjects of the first part of this experiment and those of the second part. The signs of acclimatization observed in the first part may not be a result of cold per se. Indeed, the decreased urine excretion and the return, after about five days in the cold, of the red and white blood cells as well as of the hematocrit towards normal levels may be caused by the decreased water intake paralleling the low food intake. Similarly, since a smaller subcutaneous fat thickness corresponds to a higher skin temperature (20), the gradual increase of the skin temperature in the starved subjects exposed to cold may be explained, to a certain extent, by a diminution of the fat located at the periphery. No matter what the cause may be, it seems that the starved subjects, in contrast with those fed a normal diet, have developed some acclimatization to cold.

There is a difference in the 17-ketosteroid excretion between Part I and Part II. The small amount of these steroids in the urine in the first part of this experiment was explained by the absence of proteins in the rations. In the second part, the initial increase followed by a decrease and a subsequent increase of these substances correspond respectively, accordingly to Selye (26), to the utilization of stored steroids, to a temporary hyposecretion and, finally, to a hyperactivity of the adrenal cortex.

The urinary daily excretion of ascorbic acid, in subjects receiving 525 mgm. of this vitamin, is 475 mgm. when survival rations are used and 375 mgm. with normal rations; the blood levels are respectively 1.6 and 1.2 mgm. per 100 ml. It seems that the utilization or metabolism of ascorbic acid is smaller in subjects fed a survival ration. Since the utilization of ascorbic acid is increased when the adrenal cortex is stimulated (23), this difference may be partly explained by the difference in adrenal activity as evidenced by the 17-ketosteroid excretion. If it can be assumed that the difference between intake and excretion is a measure of utilization, then this work may be interpreted as indicating that the beneficial effects of ascorbic acid, observed in men exposed to cold and fed a survival ration, are not mediated through the adrenals, but are caused by some action of this vitamin on peripheral vessels, as previously mentioned. Although the very low excretion of 17-ketosteroids, when survival rations are used, most likely indicates some adrenocortical hyposecretion, this last conclusion may not be ascertained definitely before urinary corticosteroid determinations are made.

It seems a reasonable estimate to evaluate 1 gm. of weight loss as equivalent to the production of 6.6 calories (19). Since the food intake and the weight lost or gained is known in this experiment, it is possible to calculate the daily calorie requirement of our subjects. In the second part of this experiment, the average daily food intake was equivalent to 3400 calories and the weight

gain was 80 gm. per day; the daily calorie requirement is then 2870 calories. In the first part of this experiment, the subjects consumed food equivalent to 550 calories per day and lost weight at the rate of approximately 340 gm. each day. The calculations show that the calorie requirement in this case is 2800 calories. These results show that the calorie requirement for persons living in a room at 60°F., wearing shorts and footwear only and engaged in sedentary activities, is approximately 2800 calories per day.

The oxygen consumption, as shown in the second part of this experiment, is increased by about fifteen per cent in the cold. Consequently, the extra heat produced to compensate for a drop in external temperature of 20°F. (80° to 60°F.) is estimated to be 400 calories per day. The calorie requirement for persons engaged in sedentary activities in a comfortable environment is then about 2400 calories. This last result is in agreement with the requirement prescribed by different workers (28, 22).

General Conclusion

Part I

It has been shown, when survival rations were used in the cold, that the following results were all statistically significant:

A. The supplementation of survival rations with ascorbic acid has shown some beneficial effects of this vitamin in resistance of men exposed to cold. Indeed, it has been shown that in the cold, large doses of vitamin C:

(1) increase the average skin temperature, although the internal body temperature is not changed,
(2) greatly decrease the foot trouble noticed in the low vitamin group,
(3) decrease, to a certain extent, the sensation of discomfort due to cold,
(4) maintain the blood eosinophile at higher levels.
(5) The oxygen consumption, which was measured while the subjects were comfortably warm in bed, was found to be lower in the subjects receiving larger doses of vitamin C.

B. Evidence has been given showing some acclimatization of men to low environmental temperatures. From the fifth day in the cold until the end of the experiment, we have noticed in both groups:

(1) a great reduction in cold diuresis,
(2) a significant increase in the skin temperature,
(3) a return of the leucocytes and erythrocytes to normal levels,
(4) a diminution of the discomfort caused by cold.

C. Some conclusions have also been made on the survival rations used.

(1) Survival rations, composed exclusively of carbohydrates, do not prevent the acclimatization of men exposed to cold, nor the utilization of body proteins or fats.

(2) Values for the voluntary intake of water of men in a state of semi-starvation have been obtained.

(3) Finally, the low adrenal cortex activity observed indicates a need for further investigation on the possible detrimental effect of survival rations composed exclusively of carbohydrates.

Part II

The use of normal rations in the cold leads to these conclusions:

A. Ascorbic acid was shown to have no noticeable effect on the resistance of men fed a normal ration and exposed to the low environmental temperatures mentioned.

B. No sign of acclimatization to cold was observed. The 17-ketosteroid excretion indicates, however, some adaptation of the adrenal cortex to the stressing agent imposed.

C. The average daily consumption of food was equivalent to about 3400 calories. This food was not all used as a source of energy since the subjects have gained, throughout the experiment, between two and three pounds. The results obtained with normal and survival rations have shown that the daily calorie requirement for persons engaged in sedentary activities at room temperature of 80°F. is about 2400 calories and that the corresponding values for an ambient temperature of 60°F. is approximately 2800 calories.

Acknowledgments

The authors acknowledge the co-operation of the test subjects, members of the Canadian Army and of the Royal Canadian Air Force, and the technical assistance of Miss N. Porritt and of the Food Section. We are also indebted to Dr. G. F. M. Smith and Miss M. Fleming for the statistical analyses of results.

References

1. Adolf, E. F. Advances in military medicine, Vol. II. Little, Brown Co., Boston. 1949.
2. Bacchus, H. and Toompas, C. A. Science, 113 : 269. 1951.
3. Des Marais, A. and LeBlanc, J. Can. J. Med. Sci., 30 : 157. 1952.
4. Dosne, C. Rev. soc. argentina biol. 23 : 234. 1947.
5. Dosne de Pasqualini. Am. J. Physiol. 147 : 598. 1946.
6. Drekter, I. J., Person, S., Barezak, E., and McGavack, T. H., J. Clin. Endocrinol. 7 : 795. 1947.
7. Dugal, L. P. and Thérien, M. Can. J. Research, 25 : 211. 1947.
8. Dugal, L. P. and Thérien, M. Endocrinology, 44 : 420. 1949.
9. Dugal, L. P. and Fortier, G. Federation Proc. 11 : 37. 1952.
10. Dugal, L. P. and Thérien, M. compt. rend. 234 : 245. 1952.
11. Dugal, L. P. and Thérien, M. Science, 115 : 598. 1952.
12. Farmer, C. J. and Abt, A. F. Proc. Soc. Exptl. Biol. Med. 34 : 146. 1936.
13. Folin, O. and Farmer, J. J. Biol. Chem. 11 : 493. 1912.
14. Gagge, A. F., Burton, A. C., and Bazett, H. C. Science, 94 : 428. 1941.
15. Glaser, E. M. J. Physiol. 109 : 366. 1949.
16. Glickman, N., Keeton, R. W., Mitchell, H. H., and Fahnestock, M. K. Am. J. Physiol. 146 : 538. 1946.
17. Henneman, P. H., Wexler, H., and Westenhaver, M. M. J. Lab. Clin. Med. 34 : 1017. 1949.
18. Keys, A. J. Biol. Chem. 119 : 389. 1937.

19. KEYS, A., BROZEK, J., HENSCHEL, A., MICKELSEN, O., and TAYLOR, H. L. The biology of human starvation. Univ. of Minnesota Press. 1950.
20. LEBLANC, J. Can. J. Biochem. Physiol. 32 : 354. 1954.
21. MULINOS, M. G. and POMERANTZ, L. Am. J. Physiol. 132 : 368. 1941.
22. NAT. RESEARCH COUNCIL REPRINT CIRC. Ser. No. 129. Washington, D.C. 1948.
23. PIRANI, C. L. Metabolism, 1 : 197. 1952.
24. ROE, J. H. and KEUTHER, C. A. J.B.C. 147 : 399. 1943.
25. SELYE, H., J. Clin. Endrocrinol., 6 : 117. 1946.
26. SELYE, H. Stress. Acta. Inc., Montreal, Canada. 1950.
27. STEWART, C. P., LEARMOUTH, J. R., and POLLOCK, G. A. Lancet, 240 : 818. 1941.
28. STIEBELING, H. K. and PHIPARD, E. F. U.S. Dept. of Agr. Circ. No. 507. Washington, D.C. 1939.
29. THÉRIEN, M., LEBLANC, J., HEROUX, O., and DUGAL, L. P., Can. J. Research, E, 27 : 349. 1949.
30. UNGAR, G. Lancet, 244 : 421. 1943.
31. VAN SLYKE, D. D. J. Biol. Chem. 83 : 415. 1929.

CONTINUOUS INTRAVENOUS INFUSION IN THE RAT, AND THE EFFECT ON THE ISLETS OF LANGERHANS OF THE CONTINUOUS INFUSION OF GLUCOSE[1]

By B. Kinash and R. E. Haist

Abstract

A method is described for the continuous intravenous infusion of fluids in the unanesthetized rat. When rats were infused continuously with glucose solutions for 6–14 days the total amount of islet tissue was greatly increased, as compared to that of saline-infused controls. This increase was evident also when considered in relation to pancreas weight or body weight.

The continuous intravenous infusion of glucose solutions in *guinea-pigs* was found by Woerner (12) to cause first a reduction in the granulation of the beta cells followed later by an extensive increase in islet tissue. Using larger amounts of sugar he found that the majority of the beta cells were exhausted or showed the beginning of degenerative changes. A number of different investigations concerning the effects of the continuous infusion of glucose into *dogs* have been reported. The observations include a depletion of alkali reserve and fatal acidosis (8), storage of glycogen in the liver (8, 2), reduced glucose tolerance (10), hemorrhagic destruction of the pancreas and anterior pituitary gland (8), degranulation of or damage to the beta cells of the islets (10, 3), and hyperplasia of the islets (7). Brown *et al.* (4) found that when local intra-arterial infusion through part of the pancreas was undertaken in the dog, systemic hypoglycemia and islet changes resulted. Hyperplasia in some islet cells, degranulation in others and occasionally hydropic changes were evident (4). Dohan and Lukens (5) observed that severe hydropic degeneration of the islets of Langerhans and permanent diabetes could be produced in cats by repeated intraperitoneal injections of glucose. *Rats* on a high carbohydrate diet were reported to have a greater volume of islet tissue than rats on high protein or high fat diets (9), and force-feeding large amounts of a diet high in carbohydrate has been observed to cause degranulation of the islets and islet hyperplasia (11). Though islet changes have been mentioned frequently, little quantitative data is available concerning the effects of the continuous infusion of glucose on the volume or weight of the islets of Langerhans. In the present study a method is outlined for the continuous infusion of fluids in the rat, and the effects of the infusion of glucose solutions and of physiological saline on islet weights are compared.

Materials and Methods

Method of Infusing Glucose

Female rats weighing over 150 gm. were anesthetized with ether, and the jugular vein on one side was dissected free and cannulated under aseptic

[1] *Manuscript received March 26, 1954.*
Contribution from the Department of Physiology, University of Toronto, Toronto, Ont.

conditions with a fine recurved polythene cannula. The cannula was made of polythene tubing (.025 I.D.) bent into a V shape and was inserted to the region of the superior vena cava (approximately) and tied in position with No. 4–0 silk thread. A second ligature was tied around the vessel about 1 mm. below the first and the cannula was then anchored to the underlying tissue and the tubing fixed to the skin. Sulphathiazole powder was dusted over the dissected tissues. The skin incision was sewn and the rat then was placed in a sling made of adhesive tape (Fig. 1) and put in a constant temperature box maintained at 26°C. The rats were infused with fluid by means of a roller-and-tubing type of continuous injection pump. A solution of 40% glucose in 0.5% saline, to which two Units of heparin per cc. were added, was injected at the rate of 0.8 cc. per hour for 6–14 days. In the first series glucose-injected rats and their saline-injected controls (0.85% saline) were infused at different times. In the second series control rats were injected with equivalent volumes of 0.85% sodium chloride solution at the same time, under the same conditions, and at the same rate as the glucose-injected animals. In most of the tests 150 Units of procaine penicillin G in oil were injected daily into each rat. Water and food were available ad libitum throughout the period of the test. In one experiment an attempt was made to ensure equal total caloric intakes for the glucose-injected and saline-injected animals.

At the end of the infusion period the total volume or weight of the islets of Langerhans was estimated by a slight modification of the method of Haist and Pugh (6).

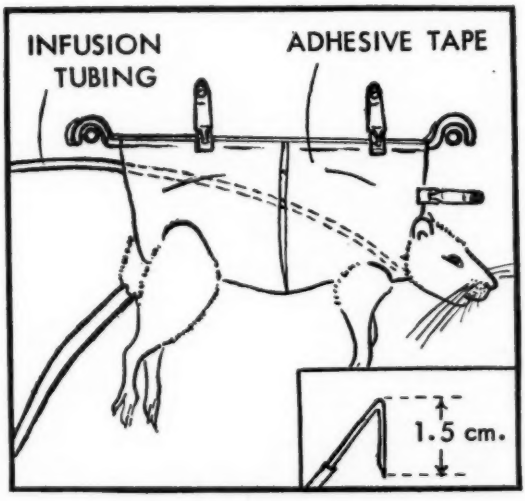


FIG. 1. Method of holding the rat in adhesive-tape slings for the infusion period. The recurved polythene cannula is shown in the insert at the lower right.

Experimental Results

The results of a series of experiments in which glucose-injected and saline-injected rats were tested separately are shown in Fig. 2. In these experiments the saline controls and the test rats were not kept under identical conditions nor infused at the same rates or for the same times. The infusion periods ranged from 4–17 days and the glucose concentrations used were 40–55% glucose in 0.5% saline. Because of the considerable range in body weights the islet weights are plotted against body weights. The values obtained in the injected groups are compared with values in a series of normal rats. It will be evident from this figure that the continuous infusion of glucose causes an increase in islet weight.

In the second experiment, glucose-injected rats and saline-injected rats were kept under the same conditions, injected at the same rates for the same times, but allowed to feed ad libitum. The results are presented in Table I.

In the third experiment, rats injected with glucose and rats injected with saline were treated as in the second experiment, but in addition an attempt was made to pair-feed the animals. Each saline-injected control received the

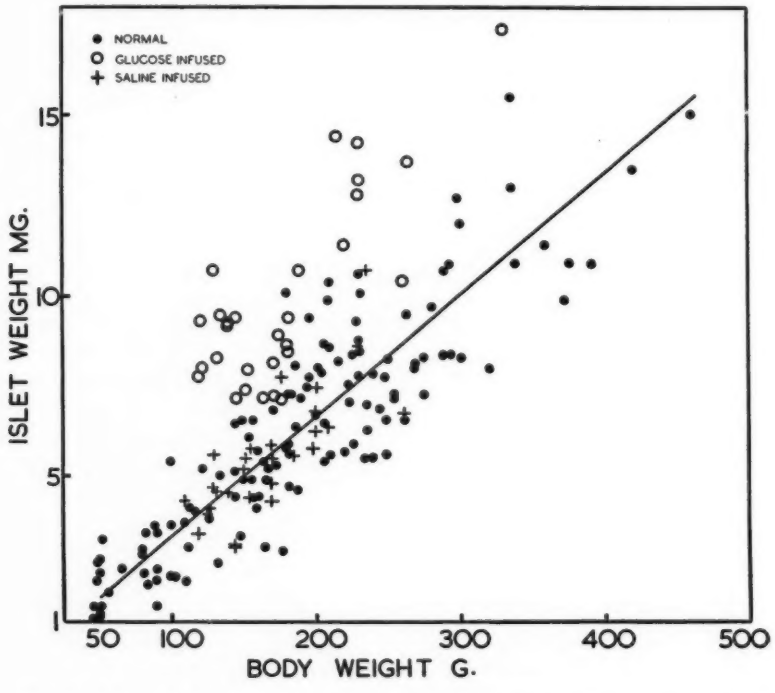


FIG. 2. Islet weight values in glucose-infused and saline-infused rats compared with those obtained in normal rats not receiving an infusion nor held in slings.

TABLE I

THE EFFECT OF THE CONTINUOUS INFUSION OF 40% GLUCOSE SOLUTIONS AND OF 0.85% SALINE SOLUTIONS INTO FEMALE RATS AT THE RATE OF 0.8 CC./RAT/HOUR. MEAN VALUES ARE FOLLOWED BY STANDARD DEVIATIONS

Group	No. of rats	Mean body weight, gm.			Mean pancreas wt., gm.	Mean pancreas wt./100 gm. final body wt.	Mean islet wt., mgm.	Mean $\frac{\text{islet} \times 100}{\text{pancreas}}$	Mean islet wt./100 gm. initial body wt.	Mean islet wt./100 gm. final body wt.
		Initial	Final	Change						
1. Glucose [fed ad lib.]	14	178 ± 20	146 ± 21	33 ± 12	0.532±0.066	0.367±0.033	8.4±0.8	1.62 ± 0.29	4.8 ± 0.7	5.9 ± 1.1
2. Saline [fed ad lib.]	14	176 ± 21	140 ± 27	36 ± 14	0.497±0.085	0.357±0.039	4.9±0.9	1.00 ± 0.12	2.8 ± 0.3	3.6 ± 0.4
3. Glucose [fed ad lib.]	15	247 ± 43	217 ± 43	30 ± 9	0.687±0.160	0.318±0.042	11.3±3.0	1.65 ± 0.23	4.6 ± 0.7	5.2 ± 0.8
4. Saline [paired-fed]	15	238 ± 28	192 ± 32	46 ± 11	0.609±0.111	0.318±0.037	6.2±1.9	1.01 ± 0.21	2.6 ± 0.6	3.2 ± 0.7
t values for 1 and 2 (by groups)		0.26	0.66	0.66	1.22	0.00	10.88***	7.32***	9.60***	7.65***
t values for 3 and 4 (by pairs)		1.76	3.76**	4.15***	3.27**	0.74	12.38***	9.0***	11.81***	10.13***

* $p < .05$ ** $p < .01$ *** $p < .001$

same amount of diet as a glucose-injected rat plus an amount equivalent in calories to the glucose which was injected each day. The results are also shown in Table I. It will be seen from the table that there is a very dramatic and highly significant increase in the weight of islet tissue in the pancreas of the glucose-injected rats as compared to saline-injected controls ($p < .001$). This is true also for the islet weight per 100 gm. body weight ($p < .001$) and for the percentage of islet tissue in the pancreas ($p < .001$). The increase in islet weight is out of proportion to any change in pancreas weight or body weight under these conditions. The body weight actually falls as a result of the injection procedure. The collected values from Table I are compared in Fig. 3.

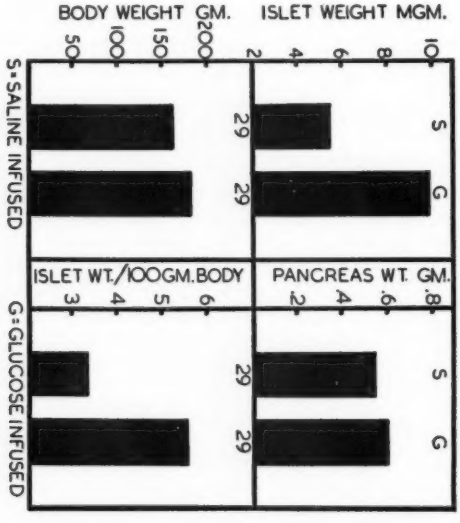


FIG. 3. Results obtained when rats were infused intravenously with glucose compared with those found when rats were infused intravenously with saline under the same conditions at the same times.

Discussion and Conclusions

The results of the present experiments support the previous evidence that the continuous infusion of glucose leads to an increase in the amount of islet tissue in the pancreas. The effect on the islets would appear to be relatively specific since proportional changes in the pancreas and the body as a whole do not occur. Indeed, the increase in islet weight occurs despite the fact that the body weight is decreasing. How the stimulating effect of glucose infusion on the islets is exerted is not yet clear. The results of Brown *et al.* (4) (*vide supra*) on the dog, and of Evelyn Anderson on the isolated perfused pancreas of the rat (1) make it seem probable that an elevation in blood sugar level can in some way directly stimulate the islet cells. In another experiment

with the continued infusion of glucose under conditions similar to those outlined above, the blood glucose levels rather soon returned to within the normal range (6–24 hr.). This adaptation may conceivably be due to stimulation of the islets and increased insulin secretion. Despite the return of the blood sugar level toward normal, the stimulation of the islets seems to have continued; hence the total amount of sugar supplied in a given period of time would appear to be of more consequence in islet stimulation than the actual concentration of sugar in the blood.

Acknowledgments

We wish to thank Prof. Charles H. Best for his continued interest in this work, and the National Research Council of Canada for financial assistance in carrying out this project.

References

1. ANDERSON, E. and LONG, J. A. Endocrinology, 40 : 98. 1947.
2. ASTWOOD, E. B., FLYNN, J. M., and KRAGER, O. J. Clin. Invest. 21 : 621. 1942. Abstr.
3. BARRON, S. S. and STATE, D. Arch. Pathol. 48 : 297. 1949.
4. BROWN, E. M., DOHAN, F. C., FREEDMAN, L. R., DE MOOR, P,, and LUKENS, F. D. W. Endocrinology, 50 : 644. 1952.
5. DOHAN, F. C. and LUKENS, F. D. W. Endocrinology, 42 : 244. 1948.
6. HAIST, R. E. and PUGH, E. J. Am. J. Physiol. 152 : 36. 1948.
7. HOUSSAY, B. A., FOGLIA, V. G., SMYTH, F. S., RIETTI, C. T., and HOUSSAY, A. B. J. Exptl. Med. 75 : 547. 1942.
8. JACOBS, H. R. and COLWELL, A. R. Am. J. Physiol. 116 : 194. 1936.
9. TEJNING, S. Acta Med. Scand. Suppl. 198. 1947.
10. VINING, K. K. J. Lab. Clin. Med. 34 : 760. 1949. Abstr.
11. WISSLER, R. W., FINDLEY, J. W., and FRAZIER, L. E. Proc. Soc. Exptl. Biol. Med. 71 : 308. 1949.
12. WOERNER, C. A. Anat. Record, 71 : 33. 1938.

THE ENZYMATIC SYNTHESIS OF CITRIC ACID BY CELL-FREE EXTRACTS OF *ASPERGILLUS NIGER*[1]

By C. V. Ramakrishnan and S. M. Martin

Abstract

Cell-free extracts of *Aspergillus niger*, N.R.C. 233, have been shown to contain the enzymes necessary to catalyze the synthesis of citrate from ATP, acetate, and oxalacetate. The "condensing enzyme", which catalyzes the condensation of acetyl-coenzyme A and oxalacetate to yield citrate, has been isolated and purified approximately 50-fold by a combination of steps involving ammonium sulphate fractionation and calcium phosphate gel adsorption.

Introduction

The "condensing enzyme" which catalyzes the reaction

$$\text{Ac–CoA} + \text{OAA} \rightleftarrows \text{citrate} + \text{CoA}^*$$

has been shown to occur in cell-free extracts of *Aspergillus niger* (14). The present communication gives further details on the isolation, purification, and properties of the enzyme. A preliminary study of some of the enzymatic reactions immediately preceding the condensation reaction is also reported.

Materials and Methods

Aspergillus niger, N.R.C. 233, was grown in a medium containing 3% malt extract, 0.5% yeast extract, and 1% glucose in shake flasks at 26°C. After 18 to 20 hr. incubation, the growth was removed, washed with water, and pressed dry on a Buchner funnel. The washed mycelium was ground in a stainless steel, planetary ball-mill (Baird and Tatlock (London) Ltd.) for 30 min. at 1°C., with an equal weight of alumina (A-301, Aluminum Ore Co.) and four times its volume of 0.02 *M* phosphate buffer, pH 7.2 (henceforth referred to as buffer). The extract was separated from the debris by centrifugation at 35,000 r.p.m. and 0°C. for 15 min.

Two hundred and sixty milliliters of the crude extract was brought to 60% saturation with solid ammonium sulphate (42 gm. per 100 ml.) and allowed to stand at 0° for a few minutes. The precipitate was removed by centrifugation at 35,000 r.p.m., dissolved in 50 ml. of buffer, and dialyzed against buffer at 1°C. for 14 hr.

The first ammonium sulphate fraction was brought to 50% saturation (35 gm. per 100 ml.), and the precipitate was removed and discarded. To the supernatant was added a further 7.0 gm. of ammonium sulphate per each original 100 ml. of solution. The resulting precipitate was removed and dissolved in 10 ml. of buffer.

[1] *Manuscript received February 26, 1954.*
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as N.R.C. No. 3304.

**Abbreviations used: Ac-P, acetyl-phopshate; Ac-CoA, acetyl-coenzyme A; CoA, coenzyme A; OAA, oxalacetate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; DPN, diphosphopyridine nucleotide; DPN ox, oxidized DPN; DPN red, reduced DPN.*

The second ammonium sulphate fraction was brought to 40% saturation (28.0 gm. per 100 ml.) and the precipitate was removed and discarded. The supernatant was brought to 60% saturation by adding a further 14.0 gm. of ammonium sulphate per each original 100 ml. of solution. The precipitate was removed and dissolved in 5 ml. of buffer.

To the third ammonium sulphate fraction was added 0.5 volumes of tricalcium phosphate gel (8.0 mgm. dry wt. per ml.). The mixture was allowed to stand at 0°C. for 10 min. and was occasionally stirred; it was then centrifuged at 35,000 r.p.m. The supernatant was removed and saved. The gel was homogenized with one volume of 0.1 *M* phosphate buffer, pH 7.2, and allowed to stand for 10 min. The eluate was removed by centrifugation.

Before it was tested for "condensing enzyme" activity, each fraction was dialyzed against buffer at 1° until most of the ammonium sulphate was removed.

Transacetylase, in the form of an ammonium sulphate fraction, was obtained from *Escherichia coli* (17). *E. coli*, N.R.C. 428, was grown in a medium containing 0.5% peptone, 0.1% yeast extract, 1% glucose, and 0.25% dibasic potassium phosphate. After 40 hr. incubation at 30°C. on a rotary shaker, the cells were harvested and washed with 0.4% sodium chloride. The washed cells were then suspended in distilled water, lyophilized, and stored over calcium chloride at 10°C. To prepare a source of transacetylase, 0.5 gm. of cells was ground in a mortar with 0.5 gm. of alumina and extracted (with grinding) with 8.0 ml. of buffer for 15 min. at room temperature. The suspension was then centrifuged at 35,000 r.p.m. for 15 min. An ammonium sulphate fraction (0–60% saturation) of the supernatant was prepared and dialyzed against buffer at 1°C. for three to four hours.

Oxalacetate, pyruvate, *l*-malate, acetate, L-cysteine, ATP (Sigma), DPN (Sigma), and CoA (Pabst) were commercial preparations and, with the exception of DPN and CoA, were used as the potassium salts. Dilithium acetyl-phosphate was prepared according to Stadtman and Lipmann (18) and tricalcium phosphate gel according to Keilin and Hartree (7).

Aconitic acid was determined at 0°C. and citric and aconitic acids at 60°C. by the methods of Saffran and Denstedt (15), acetyl-phosphate by the hydroxylamine technique (8) and total nitrogen by the micro-Kjeldahl method. Rough estimation of protein content was made by measuring ultraviolet adsorptions at 260 mμ and 280 mμ (21). The product of the enzymatic reaction was identified qualitatively by paper chromatography using *n*-butanol – formic acid – water (5 : 1 : 4) as the solvent system (9).

The assay was based on the following reactions:

$$\text{I. Ac-P} + \text{CoA} \rightleftarrows \text{Ac-CoA} + \text{phosphate (transacetylase (17))}$$

$$\text{II. Ac-CoA} + \text{OAA} \rightleftarrows \text{citrate} + \text{CoA (condensing enzyme (19))}$$

$$\overline{\text{Ac-P} + \text{OAA} \rightleftarrows \text{citrate} + \text{phosphate}}$$

One milliliter of the assay system contained: phosphate buffer (pH 7.2), 25 μM.; $MgCl_2$, 4 μM.; L-cysteine, 10 μM.; oxalacetate, 20 μM.; acetyl-phosphate, 10 μM.; CoA, 12 units; ammonium sulphate fraction of *E. coli* (9.0 mgm. protein per ml.), 0.04 ml.; "condensing enzyme" preparation, 0.2 ml. of crude extract or 0.02 ml. of fractionated preparation; water, to 1 ml. This system is the same as that used by Ochoa, Stern, and Schneider (12). Additions were made into Warburg flasks kept in ice. After addition of the enzyme, the flasks were incubated on the Warburg apparatus for 40 min. at 28°C. with the stopcocks open. At the end of the incubation period the contents of the flasks were transferred to tubes, heated in a boiling water bath for 15 min., and then cooled in ice. One milliliter of 10% trichloroacetic acid was added to each tube and the resulting precipitate was removed by centrifugation at 3,500 r.p.m. Citric (citric + aconitic) and aconitic acids were determined on the supernatant. In experiments designed to assess utilization of acetyl-phosphate, heated samples without added trichloroacetic acid were used for analysis of acetyl-phosphate. One unit of the enzyme has been defined as the amount of enzyme which, under the conditions of assay, catalyzes the synthesis of 1.0 μM. of citrate in 40 min. at 28° (cf. (12)). The specific activity of preparations is expressed as units per mgm. of protein.

Results

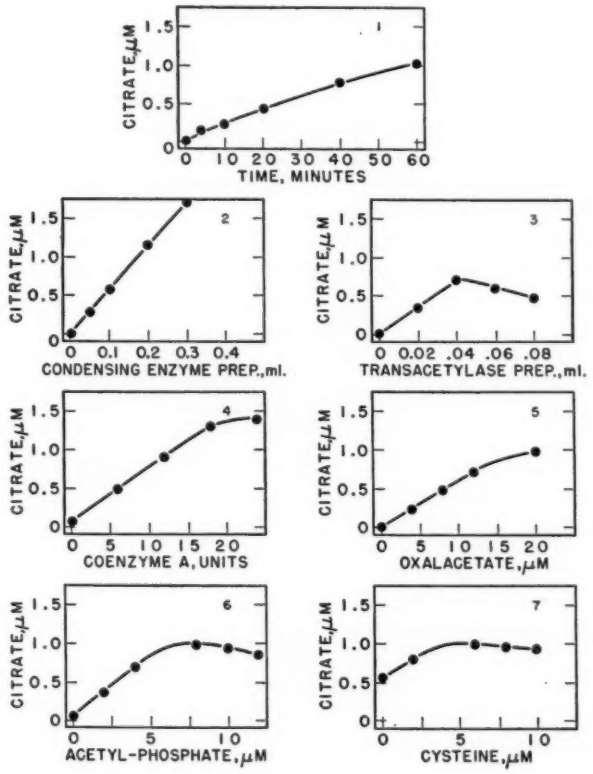
The purification of the "condensing enzyme" (Table I) resulted in a nearly 50-fold increase in specific activity in the protein of the gel supernatant. However, the 88% decrease in total units recovered indicated a considerable degree of inactivation. Increasing purification resulted in decreased stability of the enzyme during storage at −10°C. Whereas the crude extract was stable for a month or more at this temperature, the third ammonium sulphate fraction was stable for about two weeks and the gel supernatant for only one week.

TABLE I

PURIFICATION OF "CONDENSING ENZYME"

Step	Volume, ml.	Units	Protein, mgm.	Specific activity	Recovery, % of total units
Crude extract	260	1032	1638*	0.63	100
1st ammonium sulphate (0–0.6 saturation)	50	280	400	0.73	27
2nd " " (0.5–0.6 saturation)	10	269	48	5.6	26
3rd " " (0.4–0.6 saturation)	5	225	15	15.0	22
Calcium phosphate gel supernatant	14	132	4.4	30.0	12
" " " eluate	5	7	0.75	9.0	1

* *From 82 gm. wet weight of mycelium.*



FIGS. 1–7. The relation of citrate synthesis to:

FIG. 1. Time
FIG. 2. Condensing enzyme
FIG. 3. Transacetylase
FIG. 4. Coenzyme A
FIG. 5. Oxalacetate
FIG. 6. Acetyl-phosphate
FIG. 7. Cysteine

Figs. 1–7 show the relationship of citric synthesis to time and, to concentrations of enzyme, transacetylase, CoA, oxalacetate, acetyl-phosphate, and cysteine. The data in Figs. 1 and 2 were obtained using dialyzed crude extracts of *A. niger*. The remaining data were obtained with third ammonium sulphate fractions. Figs. 3, 6, and 7 indicate that supraoptimal concentrations of transacetylase preparation, acetyl-phosphate, and cysteine decreased the rate of citrate synthesis. The high rate of synthesis in the absence of added cysteine (Fig. 7) can probably be explained by the high glutathione content (10%) of the CoA preparation.

In the absence of added transacetylase (Fig. 3) there was some synthesis of citrate because of contamination of the *A. niger* fraction with small amounts of transacetylase. The presence of transacetylase was confirmed by observing the disappearance of acetyl-phosphate.

When a crude extract of *A. niger* was incubated with acetate and ATP, citrate was synthesized in the presence of oxalacetate (Table II), thus demonstrating the presence of the acetate-activating enzyme, in addition to transacetylase and the "condensing enzyme". Similarly when the extract was incubated with pyruvate, malate, DPN, acetate, and ATP, citrate was synthesized (Table II), indicating the coupling of the "acetate enzymes" with malic and lactic dehydrogenases. The data show that deletion of DPN from the assay system had no effect on the yield of citrate and that deletion of pyruvate resulted in a decrease of only 40%. These effects can probably be ascribed to the presence of DPN and pyruvate in the crude enzyme preparations.

TABLE II

SYNTHESIS OF CITRATE IN COUPLED SYSTEM

Net μM. citrate/mgm. protein*

System	Complete	Without oxalacetate	Without pyruvate	Without DPN
ATP – acetate†	0.32	0.02	—	—
Malate – pyruvate – coupled‡	0.43	—	0.26	0.43

* *System without enzyme taken as blank.*

† *Complete system contained: phosphate buffer (pH 7.2), 25 μM.; $MgCl_2$, 4 μM.; ATP, 5 μM.; CoA, 12 units; L-cysteine, 10 μM.; acetate, 20 μM.; oxalacetate, 20 μM.; crude extract of A. niger, 0.2 ml.; water, to 1.0 ml.*

‡ *Complete system contained: phosphate buffer (pH 7.2), 25 μM.; $MgCl_2$, 4 μM.; ATP, 5 μM.; CoA, 12 units; L-cysteine, 10 μM.; acetate, 20 μM.; l-malate, 20 μM.; pyruvate, 20 μM.; DPN, 0.02 μM.; crude extract of A. niger,; 0.2 ml.; water, to 1.0 ml.*

In each of the above experiments the final samples were assayed for aconitic acid but none was found. The absence of aconitic acid was also shown by the presence of only one acid (citric) when final reaction mixtures were chromatographed.

Discussion

Although many schemes have been proposed to explain the formation of citrate by *A. niger*, only those involving a C_2–C_4 condensation are compatible with the data obtained through study of isotope distribution (3, 10, 16) and of fermentation products (20). The following reactions, consistent with such a condensation have been proposed at various times:

I. acetate + oxalacetate ⟶ citrate (13),
II. acetate + malic ⟶ citrate (4),
III. acetate + succinate ⟶ citrate (2),
IV. glycolate + malate ⟶ citrate (5).

Because of the ready interchangeability of the acids involved, however, the available information has not been sufficiently discriminating to make it

possible to choose between the reactions. It has also been suggested (6) that the primary reaction product may be *cis*-aconitate rather than citrate.

The demonstration (1, 11, 19) in certain animal tissues, yeasts, molds, and bacteria of an enzyme which catalyzes the condensation of acetyl-coenzyme A and oxalacetate to yield citrate has led to the assumption that citrate is formed in this manner by *A. niger*. The recent report (14) that *A. niger* also possesses this enzyme would appear to confirm this hypothesis.

The "condensing enzyme" of *A. niger* has been shown to be quite similar to that found in animal tissues. The data presented also indicate that this organism possesses the enzymes necessary to carry out the reactions:

1. acetate + ATP $\rightleftarrows$ Ac-P + ADP
2. Ac-P + CoA $\rightleftarrows$ Ac-CoA + phosphate
3. $\underline{\text{Ac} - \text{CoA} + \text{OAA} \rightleftarrows \text{citrate} + \text{CoA}}$
   acetate + OAA + ATP $\rightleftarrows$ citrate + ADP + phosphate
4. *l*-malate + DPN_{ox} $\rightleftarrows$ OAA + DPN red
5. $\underline{\text{pyruvate} + \text{DPN red} \rightleftarrows \text{lactate} + \text{DPN}_{ox}}$
   acetate + *l*-malate + pyruvate + ATP $\rightleftarrows$ citrate + lactate + ADP + phosphate

It thus seems probable that citrate is synthesized by *A. niger*, in part at least, through a mechanism analogous to that found in other organisms.

Acknowledgment

The authors wish to acknowledge the technical assistance of Mr. J. T. Slobodian.

References

1. Barron, E. S. G. and Ghiretti, F. Biochim. et Biophys. Acta, 12 : 239. 1953.
2. Bernhauer, K. and Siebenauger, H. Biochem. Z. 240 : 232. 1931.
3. Bomstein, R. A. and Johnson, M. J. J. Biol. Chem. 198 : 143. 1952.
4. Chrzaszcz, T. and Tiukow, D. Biochem. Z. 229 : 343. 1930.
5. Ciusa, R. and Brull, L. Ann. chim. appl. 29 : 3. 1939.
6. Foster, J. W. *Chemical activities of fungi.* Academic Press, Inc., New York. 1949.
7. Keilin, D. and Hartree, E. F. Proc. Roy. Soc. (London), B, 124 : 397. 1938.
8. Lipmann, F. and Tuttle, L.C. J. Biol. Chem. 159 : 21. 1945.
9. Lugg, J. W. H. and Overell, B. T. Australian J. Sci. Research, Ser. A, 1 : 98. 1948.
10. Martin, S. M., Wilson, P. W., and Burris, R. H. Arch. Biochem. 26 : 103. 1950.
11. Novelli, G. D. and Lipmann, F. J. Biol. Chem. 184 : 213. 1950.
12. Ochoa, S., Stern, J. R., and Schneider, M. J. Biol. Chem. 193 : 691. 1951.
13. Raistrick, H. and Clark, A. B. Biochem. J. (London), 13 : 329. 1919.
14. Ramakrishnan, C. V. and Martin, S. M. Chemistry & Industry, Feb. 6 : 160. 1954.
15. Saffran, M. and Denstedt, D. F. J. Biol. Chem. 175 : 849. 1948.
16. Shu, P., Funk, A., and Neish, A. C. Can. J. Biochem. and Physiol. 32 : 68. 1954.
17. Stadtman, E. R. Federation Proc. 9 : 233. 1950.
18. Stadtman, E. R. and Lipmann, F. J. Biol. Chem. 185 : 549. 1950.
19. Stern, J. R. and Ochoa, S. J. Biol. Chem. 179 : 491. 1949.
20. Walker, T. K., Hall, A. N., and Hopton, J. W. Nature, 168 : 1042. 1951.
21. Warburg, O. and Christian, W. Biochem. Z. 254 : 438. 1932.

MODIFICATION OF PARATHION'S TOXICITY FOR RATS BY PRETREATMENT WITH CHLORINATED HYDROCARBON INSECTICIDES[1]

By W. L. Ball, J. W. Sinclair, M. Crevier, and Kingsley Kay

Abstract

Oral pretreatment of rats with chlorinated hydrocarbon insecticides greatly increases their resistance to poisoning by organic phosphate insecticides. Previous work in this laboratory demonstrated that the pretreatment is followed by a rise in the serum aliesterase level. Aldridge has shown that A-aliesterase is capable of hydrolyzing paraoxon (an organic phosphate). It is suggested that an increase in the level of this organophosphate hydrolyzing enzyme plays a part in the protection observed.

Introduction

It has recently been demonstrated in this laboratory that chlorinated hydrocarbon insecticides such as aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4,5,8-dimethanonaphthalene), dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-dimethanonaphthalene), lindane (gamma isomer of 1,2,3,4,5,6-hexachlorocyclohexane), chlordane (1,2,4,5,6,7,8 octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene), and DDT (2,2,bis-(parachlorophenyl-1,1,1,-trichlorethane) caused as much as a 30% increase in esterase levels of rat serum when administered *in vivo* (2). This finding directed attention to the possibility that compounds of this class might modify the toxicity of organic phosphates such as parathion (*O,O*-diethyl-*O*-*p*-nitrophenyl thiophosphate) long known to inhibit the cholinesterase activity of mammals (4, 6).

Experimental

Toxicity of Preparations Used

Routine LD_{50} determinations were carried out on crystalline aldrin, technical parathion*, and parathion emulsion† using groups of 50 randomized, unstarved, male and female rats. The toxicants were administered orally in corn oil. At the end of six days LD_{50} estimations were made by the method of Miller and Tainter (8). Values for these formulations are outlined in Table I.

Besides the frequently noted sex difference in the toxicity of parathion it will be observed that absorption from the emulsion is apparently greater.

In the following experiments both technical parathion and parathion emulsion were employed because it was of interest, from a practical standpoint, to know whether or not the diluents of the trade formulation played a part in the phenomenon we had observed. Preliminary investigation indicated that they had no effect and, because of its greater ease and safety of handling the emulsion was employed in most of our experiments.

[1] *Manuscript received March 29, 1954.*
Contribution from the Toxicology Section, Occupational Health Laboratory, Department of National Health and Welfare, Ottawa, Canada.

* *Technical A.A.E.E. References Standard—Lot 10126 (98.76% parathion).*

† *Prepared by Canadian Industries Limited.—Parathion Tech. 25.9%, Atlox C-1256 10.0%, Peico Hi-solv. 64.1%.*

TABLE I

LD_{50} OF FORMULATIONS OF ALDRIN AND PARATHION FOR RATS

Formulation	LD_{50} (mgm./kgm.) ± standard error	
	Male rats	Female rats
Crystalline aldrin	60.0 ± 2.4	58.8 ± 5.0
Technical parathion	9.0 ± 0.7	3.6 ± 0.3
Parathion emulsion	6.4 ± 0.4	2.6 ± 0.2

Combined Effect of Aldrin and Parathion on Rat Serum Esterase Activity

The serum esterase activity of 20 male rats was determined by an adaptation of the colorimetric method of Gomori as described in our previous paper (2). To 10 of these animals 30 mgm./kgm. of aldrin ($\frac{1}{2}$ an LD_{50}) were administered orally in corn oil. Three days later serum esterase determinations were again made on all animals and 3 mgm./kgm. of technical parathion ($\frac{1}{2}$ an LD_{50}) were given orally in corn oil to all animals. Twenty-four and 48 hr. later samples of blood were taken from the tip of the tail as described in our above-mentioned paper. After this last sampling the rats were anesthetized with ether and blood was taken from the heart to provide a check by the Warburg manometric method. The tail samples were examined with the Gomori technique and the heart samples by the Warburg method using triacetin (aliesterase sensitive) substrate as described in our paper (2). The results of the Gomori determination are presented in Fig. 1. Warburg values which

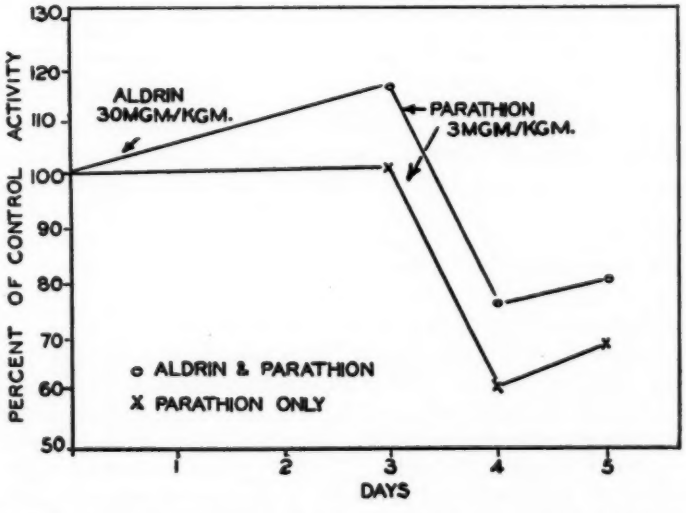


FIG. 1. Serum esterase response of aldrin-pretreated and untreated male rats to parathion.

were slightly higher are not shown. The essentially parallel fall and rise in esterase activity shown in both curves, after parathion, indicates an additive effect of the two toxicants.

Toxicity of Mixtures of Aldrin and Parathion

Table II summarizes the acute oral LD_{50} values found when mixtures of crystalline aldrin and parathion emulsion were administered simultaneously in corn oil to randomized groups of 50 female rats. Also given in the table are the values for the aldrin and parathion from which the mixtures were prepared. The calculated toxicities of the mixtures were determined by alligation (rule of mixtures) for comparison (10).

TABLE II

ACUTE ORAL TOXICITY OF MIXTURES OF ALDRIN AND PARATHION FOR FEMALE RATS

Composition			LD_{50} of mixtures as mgm./kgm. with standard errors		Significance of difference between calculated and found
Per cent parathion	Per cent aldrin	Mole ratio parathion/ aldrin	Calculated*	Found	
100	0	—	—	2.6 ± 0.1	—
80	20	4.9	3.2 ± 0.2**	3.0 ± 0.2	—
60	40	1.9	4.2 ± 0.2**	4.6 ± 0.2	$P = >0.05$
40	60	0.7	6.1 ± 0.2**	7.6 ± 0.5	$P = <0.01$
20	80	0.3	11.0 ± 0.4**	14.2 ± 0.5	$P = <0.01$
0	100	—	—	58.8 ± 2.3	—

* *Based on rule of alligation (rule of mixtures, "tea mixing" rule (10)).*
** *Standard errors for calculated values determined from distribution law of indirect measurements (11).*

Examination of the results in the table indicates that mixtures of aldrin and parathion tend to have less than the expected toxicity. That this modifying action is small is not surprising in the light of our earlier observation that, in rats treated with aldrin, the serum esterase activity did not reach a maximum until the fourth day after treatment (2) while parathion is known to cause a maximum decrease in a few hours (4,6).

Effect of Pretreatment with Aldrin on Toxicity of Parathion

To evaluate the protective action of pretreatment with aldrin against parathion poisoning, groups of 50 randomized, unstarved, male and female rats were given approximately half LD_{50} doses of aldrin (30 mgm./kgm.) orally in corn oil. On the fourth day parathion emulsion was administered orally in corn oil at several levels. The LD_{50}'s were estimated on the basis of the sixth day mortalities. The results of this experiment are summarized in Table III and indicate that aldrin pretreatment resulted in a sevenfold increase in the tolerance of the animals for parathion.

TABLE III

MODIFICATION OF ACUTE ORAL TOXICITY OF PARATHION FOR ALDRIN-TREATED RATS*

Treatment	Male		Female	
	LD_{50} mgm./kgm. ±standard error	Ratio	LD_{50} mgm./kgm. ±standard error	Ratio
Parathion	5.1 ± 0.4		2.6 ± 0.3	
Parathion following aldrin	35.9 ± 1.9	7.0	19.0 ± 1.2	7.3
Parathion	6.4 ± 0.4		3.0 ± 0.3	
Parathion following aldrin	43.9 ± 2.1	6.7	22.5 ± 1.2	7.5

**Pretreated with a single oral $\frac{1}{4}LD_{50}$ dose of aldrin (30 mgm./kgm.) four days before parathion administration.*

Because of the therapeutic potential of this protection, the experiment was repeated with technical parathion (98.76%). The protective action for both female and male rats was approximately sevenfold as before. It was also found that oral aldrin pretreatment offered the same degree of protection against intraperitoneal injections of parathion.

Protective Action of Other Chlorinated Hydrocarbon Insecticides

The protective action of chlordane and lindane at $\frac{1}{2}$ and $\frac{1}{4}$ LD_{50} levels respectively, which we have shown to cause a rise in serum esterase activity ((2), was evaluated as was that of a lower level of aldrin administration $\frac{1}{4}LD_{50}$). Table IV summarizes these results.

TABLE IV

TOXICITY OF PARATHION FOR RATS PRETREATED WITH CHLORINATED HYDROCARBON INSECTICIDES

Treatment	Male rats		Female rats	
	LD_{50}, mgm./kgm.	Ratio	LD_{50}, mgm./kgm.	Ratio
Parathion only	5.1 ± 0.4	4	3.0 ± 0.3	2.4
Parathion four days after 15 mgm./kgm. of aldrin	20.6 ± 2.1		7.1 ± 0.5	
Parathion only	—	—	3.7 ± 0.4	5.5
Parathion four days after 200 mgm./kgm. of chlordane	—		20.1 ± 1.6	
Parathion only	6.0 ± 0.5	3.2	3.1 ± 0.4	2.5
Parathion four days after 30 mgm./kgm. of lindane	19.3 ± 1.8		8.0 ± 1.1	

The apparently greater protection afforded the male rats cannot be considered significant because the effect of pretreatment is quite variable at low levels. Also this experiment was not designed to demonstrate the comparative effectiveness of these three insecticides.

Specificity of the Protective Action

Pure parathion does not inhibit chlorinesterase *in vitro* and its toxicity for warm-blooded animals depends upon its conversion by the liver to the cholinesterase inhibitor paraoxon (5). TEPP (tetraethylpyrophosphate) on the other hand requires no such conversion for its toxicity and is a strong inhibitor *in vitro*. It was of interest to see if aldrin would also protect the animals against TEPP poisoning. After using the procedure described above we found that aldrin provided about as good protection against TEPP as it did in the case of parathion—(the LD_{50} of TEPP was increased from 1.5 to 7.5 mgm./kgm. for female rats). Apparently the inhibition of the formation of paraoxon is not the explanation for the protective action.

A possible explanation for the phenomenon comes from the work of Aldridge (1) who showed that A-aliesterase is capable of hydrolyzing the phosphate group off paraoxon thus deactivating it. The same hydrolysis might occur with TEPP. An increase in aliesterase activity from the action of aldrin has already been demonstrated (2). Eserine is known to inhibit cholinesterases more strongly than it does aliesterases (9). Since structurally eserine bears no resemblance to organic phosphates, it may be unaffected by aliesterase, although we are not aware of any experimental evidence confirming this assumption. In several experiments the usual aldrin pretreatment could not be shown to offer protection against oral or intramuscular eserine poisoning.

Discussion

The fact that the chlorinated hydrocarbons must be administered several days before treatment of the rats with organic phosphates, if marked protection is to occur, suggests that the effect depends on an indirect metabolic process which appears to parallel the serum esterase rise. As the chlorinated hydrocarbon insecticides are known to act on the liver, a ready explanation of the protection appeared to be a damaged liver's inability to convert parathion to paraoxon. Modification of TEPP's toxicity appears to rule this out.

It seems more probable that the elevated aliesterase activity demonstrated in our previous paper (2) provides a reservoir of esterase which competes for hydrolysis by organic phosphates, thus sparing some of the true cholinesterase. If it is assumed that the structurally dissimilar eserine does not react with the aliesterase, an explanation of the lack of protective action in the case of eserine is available.

The five- to sevenfold decreases in the toxicities of parathion and TEPP as the result of aldrin pretreatment are arresting in the light of the comparatively small protection previously-investigated antidotes have given.

DuBois and co-workers (4) found that atropine protected dogs against two to three lethal doses of parathion. Deichmann and Rakoczy (3) evaluated buscopan (1-*n*-butyl-scopol-ammonium bromide) and found about twofold protection against both parathion and TEPP. Lewis and McKeown (7) were unable to find any single drug which was effective against even two LD_{50}'s of TEPP. When they combined scopolamine and mytolon chloride they obtained only silghtly better than twofold protection.

The practical value of a protective agent which must be administered days ahead of exposure is not in itself great. It is possible, however, that when more is learned of the mechanism of the protection other more rapidly-acting compounds may be found. The antagonism of the chlorinated hydrocarbon insecticides for those of the organic phosphate series is fortunate for agriculturists as it is common practice to work with both types in any growing season.

Acknowledgments

The authors would like to thank the following members of the Occupational Health Laboratory, Mrs. M. Keliher and Mr. E. Belanger, for their technical assistance in this work.

References

1. Aldridge, W. N. Biochem. J. (London), 53 : 110. 1953.
2. Crevier, M., Ball, W. L., and Kay, K. Am. Med. Assoc. Arch. Ind. Hyg. and Occupational Med. 9 : 306. 1954.
3. Deichmann, W. B. and Rakoczy, R., Am. Med. Assoc. Arch. Ind. Hyg. and Occupational Med. 7 : 152. 1953.
4. DuBois, K. P., Doull, J., Salerno, P. R., and Coon, J. M. J. Pharmacol. Exptl. Therap. 95 : 79. 1949.
5. Gage, J. C. Biochem. J. (London), 54 : 526. 1953.
6. Grob, D. Bull. Johns Hopkins Hosp. 87 : 95. 1950.
7. Lewis, J. R. and McKeown, W. B., Jr. Federation Proc. 12 : 342. 1127. 1953.
8. Miller, L. C. and Tainter, N. L. Proc. Soc. Exptl. Biol. Med. 57 : 261. 1944.
9. Myers, D. K. and Mendel, B. Proc. Soc. Exptl. Biol. Med. 71 : 357. 1949.
10. Smith, D. E. History of mathematics. Vol. 2. Ginn and Co., Boston. 1923–1925. p. 588
11. Tuttle, L. and Satterly, J. The theory of measurements. Longmans, Green and Company, New York. 1925. p. 219.

ACUTE ESERINE POISONING IN THE MONKEY[1]

By C. A. de Candole

Abstract

The changes in pulmonary ventilation which follow the intravenous injection of lethal and sublethal doses of eserine in the urethanized monkey have been studied. A reduction in minute volume is the outstanding change; but periods of overventilation may occur both before and after the phase of underventilation.

When death takes place within ten minutes respiration fails before the heart, whereas when death is delayed, both fail together.

Different elements of the respiratory mechanism differ in their susceptibility to eserine. Thus diaphragmatic and intercostal function are rarely lost together, and gasping may continue for long periods after eupnoeic breathing has failed.

Introduction

Many substances in use as insecticides are powerful inhibitors of cholinesterase, and therefore poisonous to man and other mammals. An accurate account of their toxic effect is a necessary basis for investigating methods of therapy. Such an account was given for several drugs of this group by de Candole *et al.* (2); they found that death in acute poisoning was due to respiratory paralysis. Eserine is the prototype of the class: it is generally supposed to kill in the same way as the other drugs but detailed information is lacking. An investigation has therefore been made of its respiratory and cardiovascular effects, emphasis being placed on the relation between sequence of events and dosage.

Methods

Monkeys (*Macaca mulatta*) weighing 2 to 4 kgm. were used; they were anesthetized with a mixture of 1% chloralose and 10 to 20% urethane given intraperitoneally (8.5–11.0 ml./kgm.). The minute volume (M.V.) was measured by having the animal breathe through suitable valves from a chain-compensated spirometer. This was done for one hour before eserine was injected and thereafter for two hours or until death. Records of breathing were made by means of pneumographs applied to the chest and abdomen and connected to bellows recorders (6). The carotid blood pressure was recorded with a mercury manometer. Eserine was injected intravenously (femoral vein) as the sulphate.

Results

(*a*) *Effect on the M.V.*

In Figs. 1, 2, and 3, the changes in the M.V. brought about by eserine are plotted as a percentage of the average M.V. measured for 60 min. before the injection. Each curve represents one experiment.

[1]*Manuscript received January 18, 1954.*
Contribution from Suffield Experimental Station, Ralston, Alta., Canada.

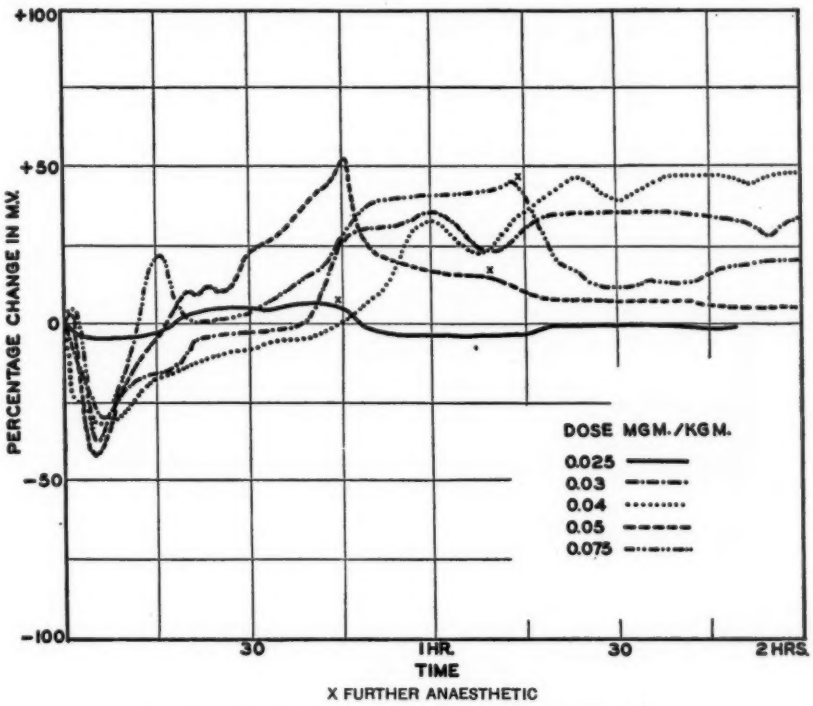


FIG. 1. Effect on M.V. of 0.025 to 0.075 mgm./kgm. eserine.

In the experiments of Fig. 1 the smallest dose (0.025 mgm./kgm.) produced no significant change, but the others all caused a temporary reduction of pulmonary ventilation, which in most instances was preceded by a transient increase. The amount of fall in M.V. varied from a quarter to nearly one-half, reaching its lowest point between the fourth and sixth minute. In all the experiments of this group, the M.V. rose again to its normal level and then, after some delay, went beyond it. Eventually it became steady at a level from 10 to 45% higher than prior to the injection, although further injections of anesthetic somewhat reduced it. Excluding the experiment at the smallest dose, if the areas above and below the zero line are compared it is clear that in every case the total overventilation was many times greater than the underventilation which preceded it.

In the experiments of Fig. 2 higher doses (0.1 to 0.3 mgm./kgm.) were given. In each case there was an immediate reduction in pulmonary ventilation (preceded in one instance by a brief rise), and in three of the six experiments the M.V. fell to zero. Return to normal after this initial fall might or might not take place. In only two out of the six experiments was the return to normal complete, the M.V. regaining its previous level and

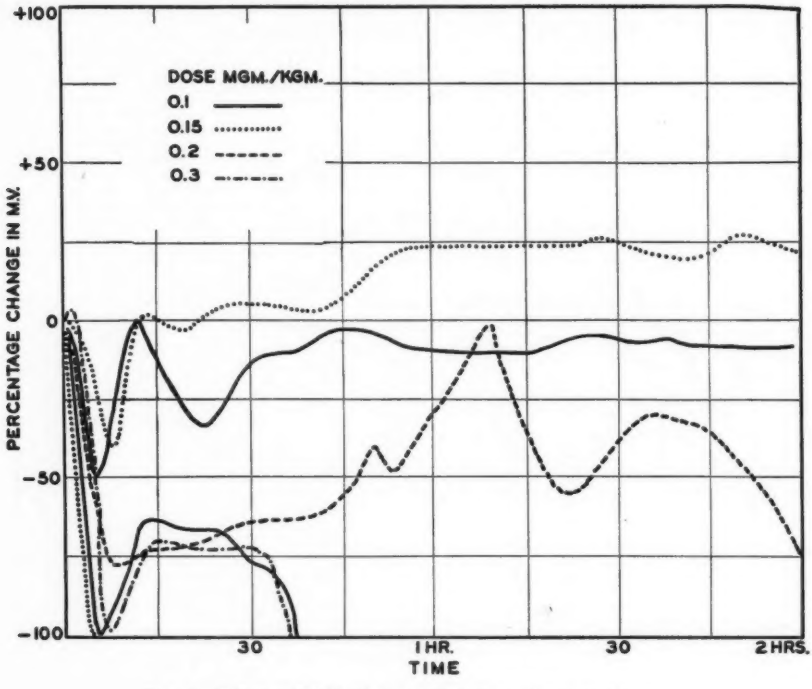


FIG. 2. Effect on M.V. of 0.1 to 0.3 mgm./kgm. eserine.

remaining high; in one of the two it became stabilized 25% higher than it was prior to the injection. In three there was a temporary return of pulmonary ventilation due to the onset of gasping, but the resulting M.V. was small and the final arrest of breathing only postponed. In the sixth experiment the M.V., after its initial fall to zero, never rose again, and all breathing ended in the third minute.

In the experiments of Fig. 3 still higher doses were given (0.4 to 2.0 mgm./kgm.) and the M.V. fell abruptly to zero in all cases. Only in a minority (two out of five) was there any return of pulmonary ventilation after the first fall, due (as in the previous group) to the onset of gasping. This, when it occurred, lasted at most three minutes.

(*b*) *Effect on the Respiratory Movements*

At doses between 0.1 and 4.0 mgm./kgm., breathing failed by stages; first normal breathing ceased; next came a stage of irregular, generally forcible, breathing; lastly gasping set in. Typically, each stage was separated from the next by an apnoeic interval. The most complete and clearly marked stage-by-stage failure occurred when the dose was 0.3 mgm./kgm. and a tracing from such an experiment is shown in Fig. 4. It shows all the features

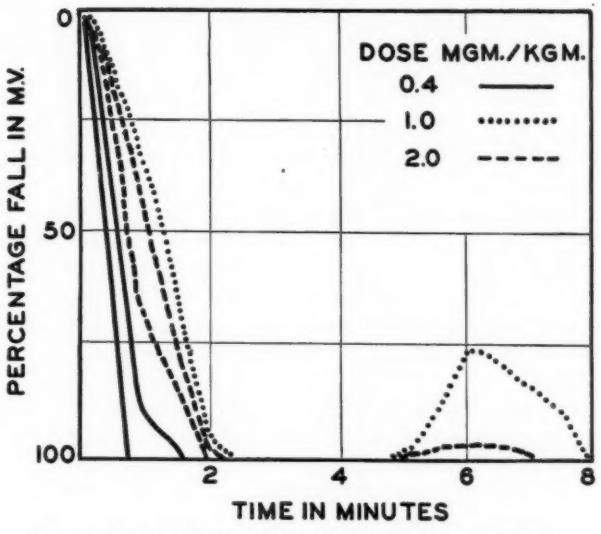


FIG. 3. Effect on M.V. of 0.4 to 2.0 mgm./kgm. eserine.

to be described, but is imperfect in that there is no distinction in the costal record between the first and second stages.

The first stage, in which breathing tapered off and came to a brief standstill, lasted from 9 to 105 sec., the time being shorter when the dose was large (Table I). After an apnoeic pause, at most 10 sec. long, this stage was followed by a second one, lasting a minute or two, in which there were rapid forcible breathing movements of uneven but typically increased strength. In some experiments these were mainly costal, and in others mainly or solely abdominal. Measurements of the M.V. showed that such movements effected little ventilation of the lungs. This second stage was succeeded, after an apnoeic interval which sometimes lasted over four minutes, by a final stage of gasping. The gasps were at a rate of two or three a minute,

TABLE I

DURATION OF STAGE ONE

Dose, mgm./kgm.	0.1	0.15	0.2	0.4	1.0	2.0	4.0	6.0
Number of experiments	1	1	4	3	16	11	2	2
Average duration of Stage 1 in seconds	105	75	94	27	26	25	20	9

(*Time measured from the end of the injection.*)

and although a good volume of air entered the lungs at each gasp, they were so infrequent that the M.V. was much below normal. Gasping lasted from two minutes to an hour or more.

The above sequence was rarely seen in full but parts of it were present in each experiment. Minor variations might be present at any dose: for example in the abdominal record of Fig. 6 there was a brief resumption of stage 2 breathing after an apnoeic pause, so that this stage was separated into two parts. Major departures from the typical sequence were, however, found both at low and high doses, the clear-cut subdivision into stages disappearing on the one hand, and the number of stages diminishing on the other. When the dose was low (i.e. between 0.1 and 0.2 mgm./kgm.), the various stages merged into one another, the first and second stages being continuous, and the second stage merged gradually into gasping without any apnoeic pause (see Fig. 5).

When, on the other hand, the dose was high (0.4 mgm./kgm. and upwards) the different stages present were sharply demarcated, but gasping (stage 3) was often absent (see Fig. 6) and, with increasing doses, stage 2 as well. As long as the dose was not above 0.2 mgm./kgm. gasping always set in; in one out of five experiments in which the dose was 0.4 mgm./kgm. it failed to occur, and, as the dose was further increased, gasping was more and more often absent, though an instance occurred where it set in after as much as 2.0 mgm./kgm. At a dose of 1 mgm./kgm. and upwards the second stage also might fail to appear, failure of breathing then taking place in a single stage (stage 1). This happened in six out of 15 experiments at 1.0 mgm./kgm., in five out of eight at 2.0 mgm./kgm., in both of two experiments at 6.0 mgm./kgm., and in a single one at 8.0 mgm./kgm.

(*c*) *Effect on the Circulation*

The classical cardiovascular effects of parasympathetic stimulation were present–bradycardia, asystole, and vasodepression–but in experiments in which respiratory failure was rapid, an adequate circulation was always maintained; after breathing ceased the heart continued to beat for several minutes, and the blood pressure record showed an asphyxial rise. When respiratory arrest was delayed, the circulation steadily deteriorated, and breathing and heart failed almost at the same time.

Discussion

The data presented here show that when sufficient eserine is injected intravenously to cause death within 10 min., the M.V. falls to zero within the first 2½ min. and from then on no more air enters the lungs, apart from negligible amounts in association with terminal gasping. To ensure death within this space of time in the monkey 2.0 mgm./kgm. or more are needed, although death sometimes comes as quickly after lower doses.

The data show, too, that after doses of eserine (0.2 to 1.0 mgm./kgm.) which cause death after a delay of more than 10 min. a profound fall in the M.V. takes place in the early stages. In these animals, however, there is

PLATE I

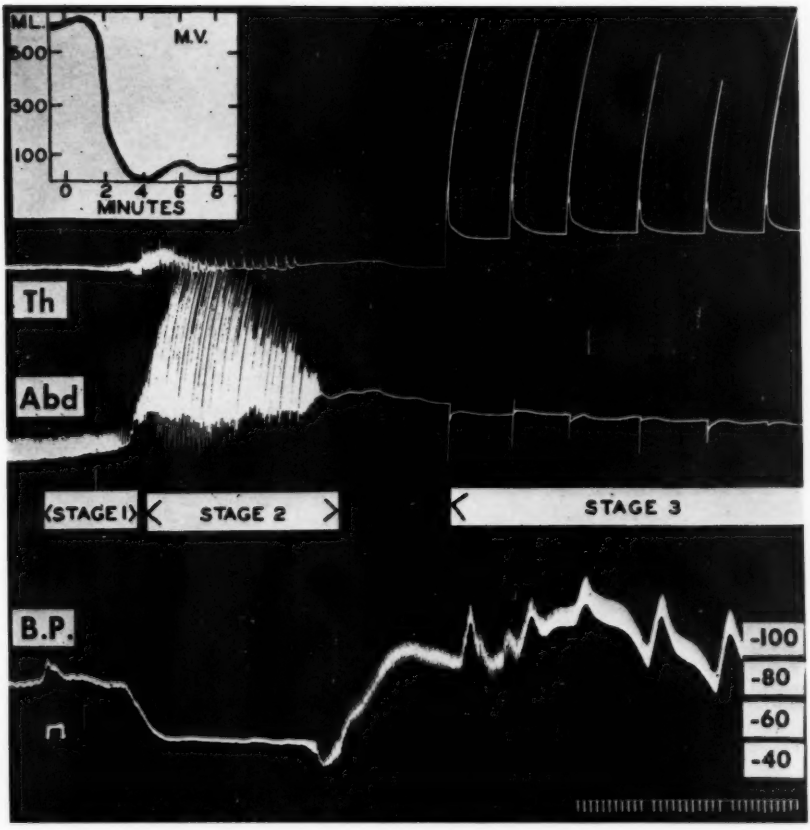


FIG. 4. Th.–costal breathing; abd.–abdominal breathing, inspiration upwards in either case; B.P.–blood pressure in mm./Hg, spirometer record inset; 0.3 mgm./kgm. eserine I.V. at the signal; time five seconds.

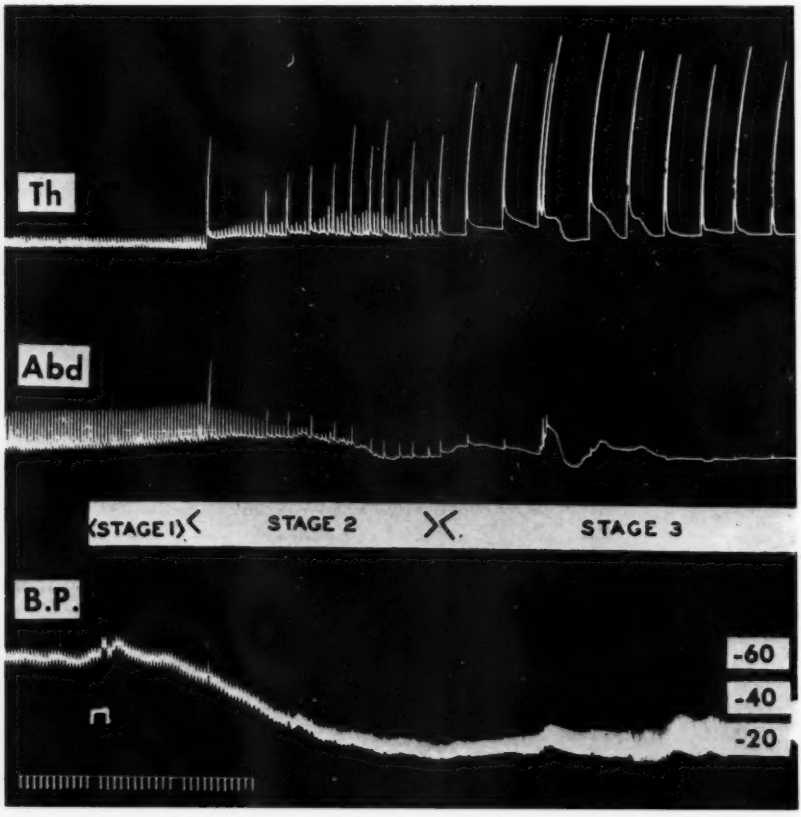


FIG. 5. Th.–costal breathing; abd.–abdominal breathing, inspiration upwards in either case; B.P.–blood pressure in mm./Hg; 0.2 mgm./kgm. eserine I.V. at the signal; time five seconds.

PLATE III

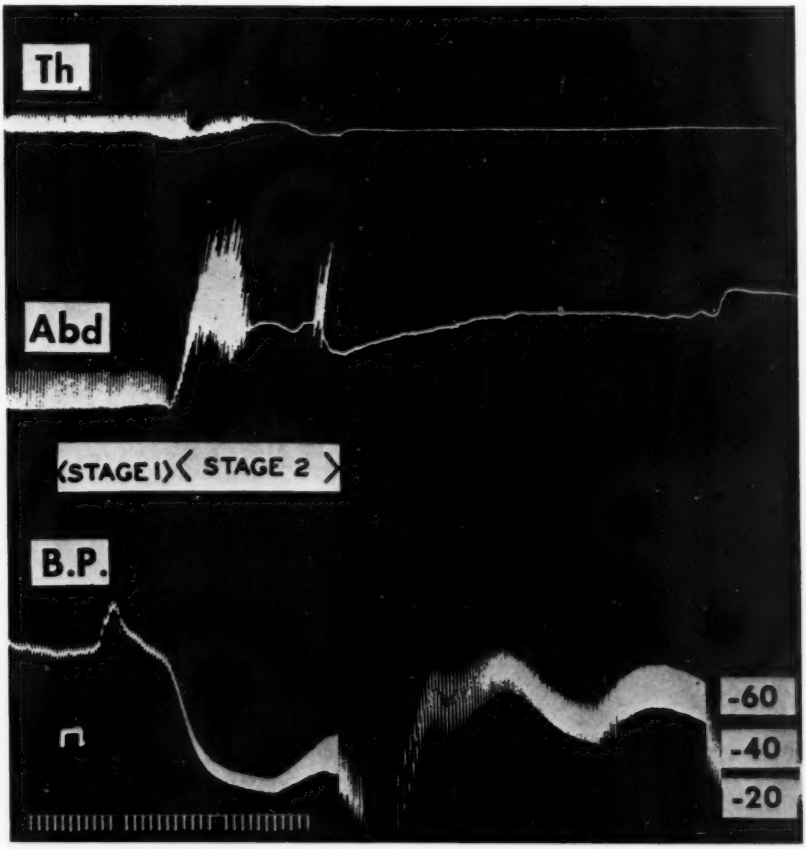


FIG. 6. Th.–costal breathing; abd.–abdominal breathing, inspiration upwards in either case; B.P.–blood pressure in mm./Hg; 0.4 mgm./kgm. eserine I.V. at the signal; time five seconds.

partial recovery as gasping sets in, which prolongs life but does not save it. After sublethal doses (i.e. in the range 0.03 to 0.15 mgm./kgm.) the M.V. falls in the early stages, but only briefly and to no great extent, a return to normal taking place within approximately 20 min. Following the return to normal, overventilation sets in, a finding previously reported by Schweitzer and Wright (5) in cats. When the M.V. during these opposing phases is compared, it becomes clear that the degree and duration of overventilation are too great to be ascribed to a 'rebound' effect of the previous underventilation. Moreover, as the present experiments confirm, overventilation also results from such doses, and indeed from somewhat higher ones, at the very outset of poisoning. Such overventilation sets in within a few seconds of an intravenous injection, but is quickly terminated in favor of the fall in M.V. which is the outstanding effect of the drug. Sublethal doses of eserine, therefore, depress respiration in some circumstances, stimulate it in others. Other authors, e.g. Gesell and Hansen (3) and Miller (4), have drawn attention to the stimulant action which eserine may have on respiration, and Schweitzer and Wright (5) gave the name 'triple response' to the sequence stimulation–depression–stimulation observed by them and confirmed here.

The present experiments show that characteristic changes in costal and abdominal breathing are associated with depression and failure of pulmonary ventilation and that these changes follow a recognizable pattern and fall into more or less well-defined stages. The interest in these changes lies in the indication they give that eserine inhibits different elements of the breathing mechanism in a selective manner, the centers responsible for gasping being more resistant than those concerned with eupnoeic breathing, and the muscles of costal breathing characteristically retaining their function longer than those of abdominal breathing. This relatively early loss of diaphragmatic function has been reported in other species and for other cholinesterase inhibitors by de Candole *et al.* (2) and by Barnes (1).

Study of the part played by the cardiovascular system confirms what every author who has worked with cholinesterase-inhibiting drugs has already noted, namely that, when death comes quickly, the circulation is still relatively unimpaired when respiration fails. The present experiments show, however, that when death is delayed it is impossible to make such a distinction, since mounting depression and final failure then involve both systems equally.

Acknowledgment

Acknowledgment is made to Miss Rose Wenner for her technical help.

References

1. Barnes, J. M. Brit. J. Pharmacol. 8 : 208. 1953.
2. de Candole, C. A., Douglas, W. W., Lovatt Evans, C., Holmes, R., Spencer, K. E. V., Torrance, R. W., and Wilson, K. M. Brit. J. Pharmacol. 8 : 466. 1953.
3. Gesell, R. and Hansen, E. T. Am. J. Physiol. 139 : 371. 1943.
4. Miller, F. R. Can. J. Research, E, 27 : 374. 1949.
5. Schweitzer, A. and Wright, S. Quart. J. exptl. Physiol. 28 : 33. 1938.
6. Wilson, K. M. J. Physiol. (London), 111 : 31 *P*. 1950.

STARCH SYNTHESIS IN *CHLORELLA VULGARIS*[1]

By J. M. Bailey[2] and A. C. Neish

Abstract

Chlorella vulgaris was found to deposit starch, in amounts up to 20% of the dry weight of the cells, when grown in a medium containing glucose. The cells did not contain cellulose or chitin. The starch was difficult to extract, being associated with an alkali-soluble, dextrorotatory, cell-wall polysaccharide. The starch, after extraction by a 26% solution of calcium chloride at 120°C., had properties quite similar to starches from higher plants. It was composed of amylose (30–40%) and amylopectin. Glucose-1-C^{14} was incorporated into the starch, by growing cells, without much breakdown and resynthesis. Cell-free extracts, obtained from the alga, contained a phosphorylase and a branching enzyme similar to those of the potato. These brought about the synthesis of an amylopectin–glycogen type polysaccharide from glucose-1-phosphate. It is concluded that the mechanism of starch synthesis in *Chlorella vulgaris* is essentially the same as in higher plants.

Introduction

Algae of the class *Chlorophyceae* are similar to the higher plants both as regards the composition of the plastid pigments (18) and the storage of starch as a food reserve (9, 10, 12). In these respects they resemble the higher plants more closely than algae of other classes. Owing to this physiological resemblance and their ready growth in pure culture, certain species of the *Chlorophyceae*, particularly members of the genera *Chlorella* and *Scenedesmus*, are being widely used for studies on photosynthesis. Consequently, further study of the storage of starch by these organisms is desirable, especially since the identification of starch in the *Chlorophyceae* is based mainly on histochemical tests and it is not certain that *Chlorella* produces a true starch.

Starch in the *Chlorophyceae* is usually closely associated with the pyrenoids. Some workers thought that starch was formed from the pyrenoid material, or in the pyrenoid, while others showed that it was formed in the chromatophores. The cytological work leading to these views has been reviewed by Bold (10.) There is some question whether starch synthesis in *Chlorophyceae* follows the same course as in higher plants, where pyrenoids are absent. This paper describes experiments which show that *Chlorella vulgaris* can produce a starch like that of higher plants by a mechanism essentially the same.

Experimental and Results

Materials

α–Glucose-1-phosphate (potassium salt) and potato amylose were obtained from Prof. S. Peat, Department of Chemistry, University College of Wales, Bangor. The amylose had a blue value (11) of 1.37 and an iodine binding of

[1] *Manuscript received March 17, 1954.*

Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan. Issued as Paper No. 170 on the Uses of Plant Products, and as N.R.C. No. 3303.

[2] *National Research Council of Canada Postdoctorate Fellow, 1952–53.*

18.3%, measured in 0.5 *N* potassium iodide by the method of Bates *et al.* (7). The wheat starch sample (blue value 0.51) was supplied by Ogilvie Flour Mills Co., Fort William, Ont. as "unmodified" wheat starch. Amylose and amylopectin were prepared from it by butanol precipitation (28, 33). The "Lintner" soluble starch was a commercial sample supplied by Merck and Co., Montreal. β–Amylase was prepared from soya beans by the method of Peat *et al.* (25). The sample (activity, 4600 units per gm.) was free from α–amylase and maltase. α–Amylase was made from human saliva (one liter) by the method of Meyer *et al.* (20). The final crystallization stage was omitted and the enzyme was freeze-dried in citrate buffer (0.2 *M*, pH 6.5); the product had no maltase activity. Glucose-1-C^{14} was synthesized by the method of Isbell *et al.* (17).

Methods

Reducing sugars were determined by the volumetric method of Schaffer and Hartmann (29) and total carbohydrates in holocellulose by a colorimetric method using anthrone (21). Both procedures were standardized against glucose, and the Shaffer–Hartmann procedure against maltose as well. Inorganic phosphate was determined by Allen's method (3), a correction being applied for the hydrolysis of glucose-1-phosphate, when necessary. Carbon-14 was measured in a gas-flow counter working in the proportional region. This apparatus was standardized against the U.S. National Bureau of Standards C^{14} standard, a sodium carbonate solution rated at 1280 dps./ml.

Mixtures of monosaccharides with oligosaccharides and salts were fractionated by chromatography on charcoal (Darco G 60) columns (31, 32). Mixtures of monosaccharides were resolved by paper chromatography, on Whatman No. 1 and No. 54 papers, by the descending solvent technique. Chromatograms were developed with ethylacetate – aceticacid – water (3 : 1 : 3) and the sugars were detected by using aniline phthalate (23). It was found to be advantageous to remove salts and other impurities from sugar solutions, prior to paper chromatography, by passage through a small charcoal column. In this way it was found that 10 mgm. of monosaccharides could be separated from 500 mgm. of salts, the salts (sulphates, acetates, chlorides, or citrates of sodium and potassium) passing through first on elution with water.

Starch Formation in Chlorella vulgaris

Chlorella vulgaris was grown in Emerson's medium with added glucose as described previously (22). The amount of growth was measured by determining the volume of packed cells deposited from 10 ml. portions of the culture centrifuged under standard conditions (22). The starch content of the cells was measured as follows: The sedimented cells were transferred to a 25 ml. Erlenmeyer flask with the aid of 2–4 ml. water. Calcium chloride solution (6 ml., 46%) was added and the starch was extracted by autoclaving at 120°C. for one hour. The extract was centrifuged and the supernatant made

up to 10 ml. with water. A portion (1–3 ml.) was treated with iodine (0.5 ml., 0.2% in 2% potassium iodide) and water to give a volume of 50 ml. The intensity of the blue coloration, at 680 mμ, was used to calculate the starch content. It was found that the blue value (0.55) of the extracted starch was independent both of the age of the culture and the glucose content of the medium.

TABLE I

STARCH FORMATION BY *Chlorella vulgaris*

Grown at 30°C. in illuminated incubator (200 ft-c.)

Days growth	Packed cells (ml./liter)	Starch (mgm./liter)	Starch* (per cent)	Glucose† concentration
0	—	—	1.4‡	1.00
3	7.3	315	17.2	0.56
5	10.2	330	12.9	0.30
7	12.4	225	7.3	0.13
13	15.4	140	3.6	0.00

* *Based on dry weight of cells.*
† *As per cent in the medium; used Emerson's medium plus one per cent glucose.*
‡ *% Starch in cells used as inoculum determined on cells grown in Emerson's medium without added glucose.*

The variation of starch content with the age of the culture is shown in Table I. It reaches a maximum and then decreases as the concentration of glucose in the medium becomes low. The starch content of cells was increased to a maximum of 20–25% by increasing the concentration of glucose in the medium to 1.5%, but further addition of glucose, even up to 6%, did not affect the starch content of cells. Although growth is slower in darkness, it was found that the starch content was not much different from that of cells grown in illuminated cultures under conditions otherwise the same. The blue values of two starches extracted from cells grown in darkness were, however, significantly higher (0.61 and 0.91).

The rate of starch formation in cells, cultured without added glucose was very low, being approximately 0.1 mgm./liter/day. This was increased about 1000 times (100 mgm./liter/day) by addition of glucose to the medium. This made possible the production of enough starch for isolation and study.

Isolation of Starch from Chlorella vulgaris

The organism was cultured in light in a series of 1-liter Erlenmeyer flasks each containing 100 ml. of Emerson's medium with glucose (1%). The cells were harvested, by centrifugation, after five to seven days of growth, when the starch content was near its maximum. They were washed once with water

and then twice with a mixture of equal volumes of ethanol and ethyl ether in the centrifuge tube. The residue was air-dried; the yield of dried cells was usually 3–4 gm. per liter of medium. The starch was isolated by a modification of a method used for leaf starches (13). Dried cells (2–3 gm.) were extracted with methanol in a Soxhlet extractor and the residue dispersed in 80 ml. of water. Calcium chloride (130 ml., 46%) was added and the mixture autoclaved at 120°C. until the cells no longer contained iodine-staining material (usually one hour). The cellular residue was removed by centrifugation and the starch precipitated from the extract by addition of *N* hydrochloric acid (18 ml.), 20% sodium chloride (36 ml.), and iodine solution (9 ml. containing 1.08 gm. of iodine and 1.80 gm. of potassium iodide).

The starch–iodine complex was centrifuged out and decomposed by treatment with 30 ml. of ethanolic sodium hydroxide (0.25 *N*). The crude starch was then washed once with 60% ethanol in water and dissolved in aqueous sodium hydroxide (0.25 *N*). The solution was neutralized with sulphuric acid (10 *N*) to the phenolphthalein end point, filtered, and the filtrate dialyzed exhaustively against distilled water to remove salts. It was then emulsified with toluene to remove traces of protein at the interface. The starch was precipitated from the aqueous layer by addition of two volumes of methanol and dried by washing with ethanol and ether. The yield was 200–300 mgm. of dry material. This starch was soluble in hot water, giving a viscous paste at 2% concentration. It gave 90–95% glucose on hydrolysis as determined by the method of Pirt and Whelan (27).

Hydrolysis of Chlorella Starch by α-Amylase

A starch sample (115 mgm.) was prepared by repeated extraction of 1 gm. of methanol-defatted cells with calcium chloride under the conditions described previously (13) for extraction of leaf starches. This sample was treated with α-amylase until the iodine stain was negative and the concentration of reducing sugars had reached a maximum value. The mixture of sugars thus obtained was fractionated on a charcoal column, 1.6 × 18 cm. No monosaccharides were found after eluting with 600 ml. of water but 37 mgm. of a reducing disaccharide was removed by 7.5% ethanol (135 ml.) and 19.6 mgm. of a reducing trisaccharide by 15% ethanol (195 ml.). Both of these fractions were chromatographically homogenous (on paper) and gave glucose as the only product on acid hydrolysis. The disaccharide had a molecular weight, from reduction of the Shaffer–Hartmann reagent (5), of 332, $[\alpha]_D$ 134°, and an R_g value of 0.52. The corresponding values for maltose are 340, 137°, and 0.51. The trisaccharide had molecular weight 497, $[\alpha]_D$ 176°, and R_g value 0.46 (maltotriose: 504, 160–166°, 0.45). In addition the disaccharide gave an osazone with the same crystalline form and decomposition point (192–194°C.) as maltosazone.

Comparison of Chlorella, Wheat, and Potato Starches

The colored products formed by interaction of starch and iodine were compared (19) using starches obtained from *C. vulgaris* and from higherplants.

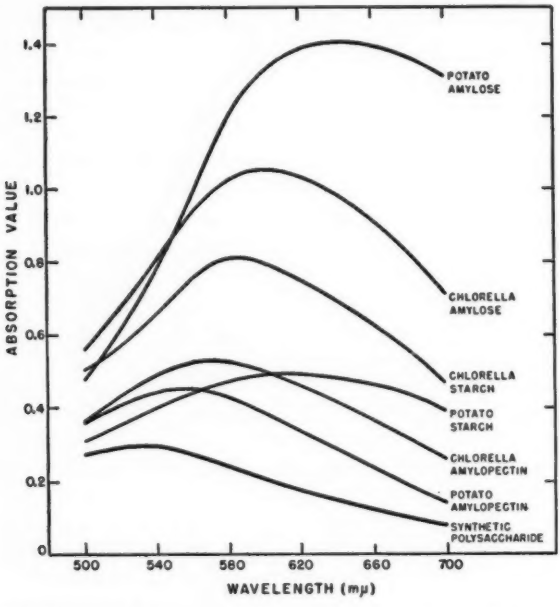


FIG. 1. Light absorption by starch–iodine complexes. The synthetic polysaccharide was prepared from glucose-1-phosphate, using an enzyme preparation from *C. vulgaris*, as described in footnote to Fig. 4.

The starch was dispersed in aqueous sodium hydroxide (0.25 *N*). The solution was neutralized with sulphuric acid and a portion equivalent to 1 mgm. of starch was treated with a solution containing 2 mgm. of iodine and 20 mgm. of potassium iodide in a final volume of 100 ml. The light absorption was measured at 680 mμ using a Coleman Junior Spectrophotometer. Blue values (11) were calculated by comparison with the potato amylose sample. Further comparisons were made by plotting the absorption values from 500–700 mμ. The iodine stain of the starch from *C. vulgaris* was similar to that of potato starch (Fig. 1) with a higher blue value (0.55) and a maximum light absorption at 490–500 mμ. This blue value corresponds to an amylose content of 32%, assuming the algal starch to be a mixture of amylose (blue value, 1.45) and amylopectin (blue value, 0.1).

Iodine binding was also measured by potentiometric titration, essentially as described by Bates *et al.* (7). The starch (15–25 mgm.) was dissolved in 5 ml. of potassium hydroxide (0.5 *N*), the solution neutralized by hydriodic acid (0.5 *N*), the volume adjusted to 50 ml., and the solution titrated with iodine (0.001 *N*) in aqueous potassium iodide (0.5 *N*). The E.M.F. of a cell formed between a calomel (saturated potassium chloride) electrode and a platinum electrode immersed in the solution was measured by a Leeds and Northrup type K potentiometer. Amylose contents were calculated by

comparison of the amount of iodine bound with the amount bound by the potato amylose sample. The curve obtained (Fig. 2 A) was intermediate between that for amylose and amylopectin and did not show the inflection normally associated with the amylose component. A similar potentiometer titration curve was given by Lintner soluble starch (Fig. 2 A). A sample of wheat amylose degraded to an average chain length of 60–80 units by acid hydrolysis was found to bind iodine at a potential similar to that of chlorella starch (Fig. 2 B).

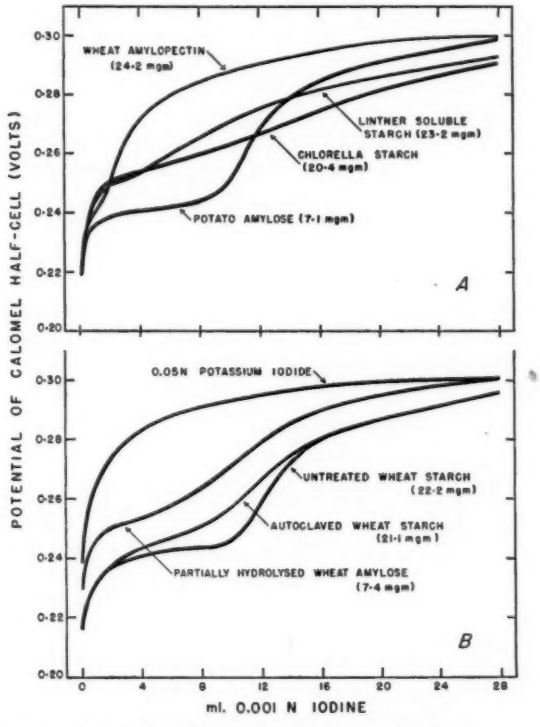


FIG. 2. Iodine titration curves of starch fractions.

The amylose content of the starch isolated from *C. vulgaris*, calculated from the potentiometric titration, was 40.5%, a figure considerably higher than that (32%) obtained from the blue value. This discrepancy is partly due to changes induced in the starch during its isolation. This was shown by subjecting a sample of wheat starch to the same conditions, i.e., autoclaving for two hours at 120°C. in calcium chloride (20%). Before treatment the wheat starch had an amylose content of 29% from measurement of the blue value and 31% by titration. After treatment these values were 22.8% and 34.6% respectively and the shape of the titration curve was changed (Fig. 2 B).

β-amylase from soya beans was found to act on chlorella starch with production of maltose and residual dextrins. A portion of the dried starch (30 mgm.) was dispersed in 15 ml. of sodium hydroxide (0.25 *N*). The solution was neutralized with sulphuric acid (10 *N*) and 400 units of soya bean β-amylase (dissolved in 5 ml. of 0.2 *M* acetate buffer of the appropriate pH) were added. The volume was adjusted to 36 ml. by addition of water and the mixture was incubated at 35°C. A blank in which the polysaccharide was omitted was made up in the same way. Portions (5 ml.) were withdrawn periodically for determination of liberated maltose until this reached a limiting value. The residual dextrin was then treated with iodine for determination of the blue value. At pH 4.8 the conversion to maltose was 61% and the residual dextrin has a blue value of 0.14. This indicates the presence of α-1,6 branch linkages of the amylopectin type. The conversion into maltose at pH 3.6 (where the β-glucosidase in the β-amylase preparation is inactive (26)) was 57% and the residual dextrin had a blue value of 0.315. This suggests that β-linkages, similar to those in the amyloses of higher plants (26), are also present in chlorella starch.

A small sample of starch (30 mgm.) isolated from *C. vulgaris* was fractionated into amylose and amylopectin by butanol precipitation (28, 33). The amylose fraction (8 mgm., 27%) gave an intense blue coloration with iodine (blue value 0.81, max. 610 mμ). The butanol complex was not crystalline. The amylopectin fraction (18 mgm., 60%) gave a red color with iodine (blue value 0.31, max. 570 mμ). The light absorption curves are shown in Fig. 1 along with those of corresponding fractions of potato starch.

Starch-synthesizing Enzymes of Chorella vulgaris

Freshly harvested, wet packed cells (7 gm.) were slurried in a mixture of 1% sodium chloride (5 ml.), toluene (2 ml.), and water (10 ml.). The mixture was shaken at 32°C. for 30 min. and then subjected to sonic treatment in a Raytheon model DF-101 oscillator (200 watt, 10 kc.) for one hour. After addition of 100 ml. of ice-cold water the mixture was clarified by centrifugation at 0–4°C., followed by filtration through a Whatman No. 5 paper at 0°C. The cell-free extract obtained was used to demonstrate the presence of a phosphorylase and a branching enzyme (Q-enzyme). *C. vulgaris* was rather resistant to most grinding, sonic, and autolyzing treatments, used separately, but almost complete disintegration of the cells was obtained by sonic treatment in the presence of autolyzing agents, as used above.

Phosphorylase activity was demonstrated by incubating the cell-free juice with glucose-1-phosphate and primer. Inorganic phosphate was liberated (Fig. 3) and a polysaccharide of the amylopectin–glycogen class was formed which gave a red-purple color with iodine (Fig. 1). This synthetic polysaccharide was degraded at pH 4.8 with β-amylase and the formation of a residual dextrin detected by iodine staining. From this the presence of branching in the synthetic polysaccharide may be inferred. The phosphorylase is similar to plant, rather than animal, phosphorylases in being primed almost as efficiently (60%) by a short chain dextrin (amylopentaose) as by

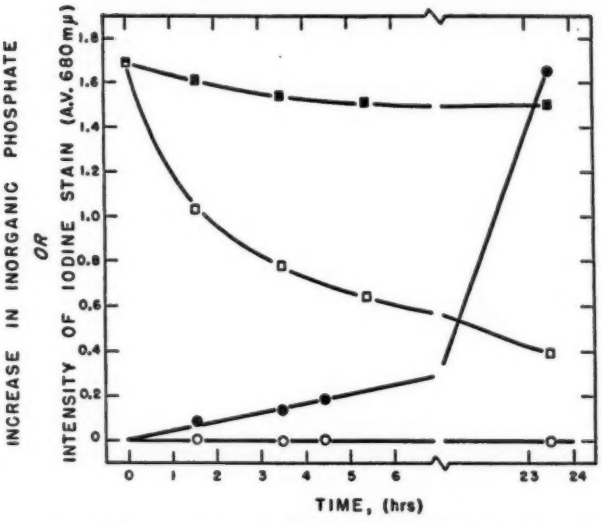


FIG. 3. Action of cell-free extracts of *C. vulgaris* on soluble starch and on glucose-1-phosphate. Each digest (10 ml.) contained cell-free extract (2 ml.) and 0.2 *M* acetate buffer at pH 6.5 (2 ml.). Other additions were as follows:
Filled circles: 0.1 *M* glucose-1-phosphate at pH 6.5 (1 ml.) and primer (0.1 mgm.).
Open circles: same as filled circles plus *M*/6500 mercuric chloride (1 ml.).
Open squares: 1% soluble starch (2 ml.).
Filled squares: same as open squares plus *M*/6500 mercuric chloride (2 ml.).
The digests were incubated at 35°C., portions (0.5 ml.) being withdrawn periodically for iodine staining (squares) or for determination of inorganic phosphate (circles). The primer was a mixture of shortchain dextrins obtained by hydrolysis of potato amylose to the achroic point (5). The ordinate is in arbitrary colorimeter units.

soluble starch (6). It was more sensitive to certain inhibitors; 17% inhibition was brought about by 0.6% ammonium molybdate and 60% by *M*/260,000 mercuric chloride (16). The pH optimum measured in citrate buffer with soluble starch as primer was 5.9–6.2 at 35°C. (Fig. 4). The phosphorylase content of *C. vulgaris* is comparable with that of higher plants. The yield was about 1.2 units per gm. of wet, packed cells, the corresponding figures for bean seeds and potato tubes being 0.5 and 1 unit, respectively (16).

The maximum rate of starch synthesis observed (100 mgm./liter/day) would require 0.4 units of phosphorylase working under optimum conditions. The yield of phosphorylase (*ca.* 18 units/liter of culture), is sufficient to account for all the starch synthesis in *Chlorella vulgaris*.

The presence of a branching enzyme similar to Q-enzyme (24) was detected by the reduction in the iodine coloration of a soluble starch and of potato amylose, brought about without appreciable liberation of reducing groups (Table II and Fig. 3). The action of this branching enzyme was inhibited by *M*/32,500 mercuric chloride which shows that the color changes were not caused by complexing of amylose with fatty acids (8). The enzyme had an optimum pH of 7–7.3 when measured in veronal–hydrochloric acid buffers, containing amylose, at 25°C.

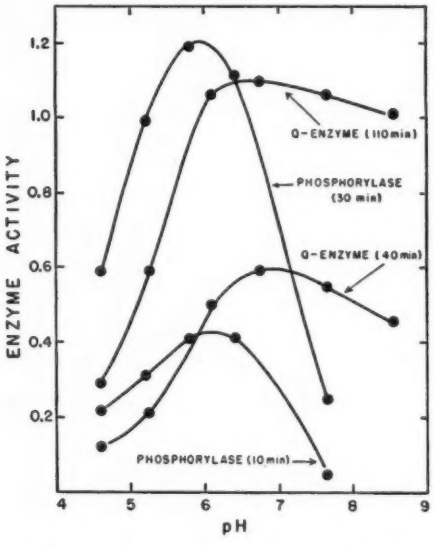


FIG. 4. Activity of phosphorylase and Q-enzyme of *C. vulgaris* at various hydrogen ion concentrations. The enzyme solution (20 ml.) contained the protein precipitated from 35 ml. of a cell-free extract between 20 and 30% concentration (w/v) of ammonium sulphate. Q-enzyme digests contained enzyme solution (1 ml.), 0.2% amylose solution (2 ml.), and veronal–hydrocloric acid buffer (2 ml.). They were incubated at 25°C., portions (1 ml.) being withdrawn for iodine staining at 0, 40, and 110 min. Phosphorylase digests contained enzyme solution (1 ml.), 1% soluble starch (0.5 ml.), 0.1 *M* glucose-1-phosphate (0.5 ml.), and 0.2 *M* citrate buffer (1 ml.). Incubation was at 35°C. Portions (1 ml.) were withdrawn for determination of inorganic phosphate liberated at 10 and 30 min. Phosphorylase activity is expressed in terms of inorganic phosphate liberated, and Q-enzyme activity as the reduction in the iodine stain of the amylose (measured at 680 mμ). The ordinate is in arbitrary colorimeter units.

TABLE II

ACTION OF CHLORELLA Q-ENZYME

% Of initial iodine coloration (680 mμ)	% Reducing sugar formed (as maltose)		
	Chlorella Q-enzyme*	Potato Q-enzyme†	α-Amylase†
85	0	0	5.25
13	3.75	2.0	18.5
7	4.50	2.75	22.0

* *The cell-free extract (5 ml.) was mixed with 15 ml. of a 0.5% solution of potato amylose and 5 ml. of 0.2 M acetate buffer at pH 6.5. The mixture was incubated at 25°C.*

† *See reference (4).*

An attempt to separate the phosphorylase and Q-enzyme of *C. vulgaris* by ammonium sulphate precipitation (Table III) gave little fractionation. However ammonium sulphate precipitation was found to be a convenient method of concentrating the enzymes. The precipitates could be freeze-dried in citrate buffer (0.2 *M*, pH 6.5) without loss of enzyme activity.

TABLE III

AMMONIUM SULPHATE PRECIPITATION OF CHLORELLA PHOSPHORYLASE AND Q-ENZYME

Concentration of ammonium sulphate*	% Of total enzyme activity in precipitate	
	Phosphorylase	Q-enzyme
0–20	0	0
20–25	69	57
25–30	29	27
30–35	2	8
35–40	0	7

* % *Weight/volume* (*pH 7.0*).

Investigation of the "Holocellulose" of Chlorella vulgaris

Three liters of a culture of *C. vulgaris* (grown for seven days with illumination in Emerson's medium enriched with 1% glucose) was treated with 15 ml. of glacial acetic acid. The cells were centrifuged down and washed with water to give 46 gm. of wet, packed cells. This fraction was washed twice in the centrifuge with 500 ml. portions of a mixture of equal volumes of ethanol and ether. The air-dried residue (12 gm.) was slurried in water (65 ml.), heated to 75°C., and glacial acetic acid (0.90 ml.) and sodium chlorite (10 gm.) were added. After one hour at 75°C. the cells were centrifuged, washed with water, and treated with acid chlorite again in the same manner. After three chlorite treatments the residue was washed in the centrifuge with successive 250 ml. portions of water, methanol, ether, and then air-dried.

The crude "holocellulose" (5.4 gm.) thus obtained was a pale yellowish powder containing 0.2% ash and 1.68 Kjeldahl nitrogen. The nitrogen content was attributed to denatured protein rather than chitin, since a sample hydrolyzed by five times its weight of concentrated hydrochloric acid for 2.5 hr. at 90–100°C. gave less than 1% of glucosamine as measured by the method of Elson and Morgan (14). The pentosan content was low, only 2.26% furfural being obtained following the colorimetric procedure of Adams and Castagne (2). The "holocellulose" consisted mainly of carbohydrate, however, since on treatment with the anthrone reagent it gave 66% of the color given by an equal weight of glucose. Hydrolysis with 1.5 *N* sulphuric acid at 100° for two hours liberated 51% of reducing sugars (determined as glucose).

Microscopic examination of the "holocellulose" showed that it was composed of particles the same size and shape as the original cells. These particles stained a deep blue when treated with iodine, thus indicating the presence of starch. When the crude "holocellulose" (4.0 gm.) was autoclaved with 56 ml. of aqueous potassium hydroxide (10%), at 120°C. for 16 hr., it all dissolved except 0.16 gm. (4%). The solution was neutralized with glacial acetic acid and the precipitate which formed was discarded. Treatment of this clear neutral solution (350 ml.) with ethanol (700 ml.) caused precipitation of a hemicellulose fraction (2.23 gm.) which contained only 0.05% Kjeldahl nitrogen but still gave a deep blue color with iodine.

This hemicellulose fraction (1.5 gm.) was dissolved in 50 ml. 0.2 *N* sodium hydroxide and neutralized to pH 6.5 by addition of sulphuric acid and acetate buffer. The starch was removed by treating this solution with α-amylase (50 mgm. of freeze-dried preparation) for 8.5 hr. until the reducing power of the digest was constant and a color was no longer formed with iodine. The enzyme was deactivated by heating and the coagulated protein filtered out. The solution and washings (70 ml.) were dialyzed overnight against two changes of distilled water (4 liters). The polysaccharide was precipitated from the sugar-free solution by the addition of three volumes of ethanol. The product was dried by grinding with ethanol and washing with ether. It was recovered as a cream colored powder (0.69 gm.) representing 45% of the hemicellulose. This polysaccharide was readily soluble in hot water giving a faintly opalescent light brown solution; $[\alpha]_D$ 61.5° (*c.* 1.08) following decolorization with charcoal. Hydrolysis for two hours with sulphuric acid (1.5 *N*) at 100° gave 81.7% of reducing sugars (determined as glucose). Examination of the hydrolyzate by paper chromatography indicated a component with the same R_f as glucose to be the major product, and smaller amounts of two other components having the same R_f values as rhamnose (or fucose) and xylose respectively.

Incorporation of D-*Glucose-1-*C^{14} *into Starch by Chlorella vulgaris*

The organism was grown under illumination in Emerson's medium enriched with D-glucose-1-C^{14} having a specific activity of 72.3 μc. per gm. of carbon-1 carbon (12.05 μc. per gm. of total carbon). The cells were harvested by centrifugation, and a "holocellulose" fraction was prepared from them by three successive chlorite treatments, as described above. This fraction contained a considerable amount of starch. It was treated with α-amylase, dialyzed, and the sugars passing through the membrane were isolated by chromatography on charcoal. A fraction containing 65 mgm. of a mixture of maltose and maltotriose was obtained by elution with 15% ethanol. This fraction was hydrolyzed to glucose by acid (27). The glucose was purified by chromatography on a second charcoal column and crystallized after addition of nine parts of inactive glucose. The yield was 165 mgm. having a specific activity of 1.175 μc. per gm. of total carbon. When corrected for the carrier added this corresponds to 11.75 μc. per gm. of total carbon for the glucose in the starch molecule (97% of the original activity).

The activity in carbon-1 was determined by the method of Sowden (30) as modified by Abraham *et al.* (1). It was found to be 85% of carbon-1 of the glucose added to the culture, after correction for the carrier added. This procedure was standardized by degradation of a sample of the original glucose.

Discussion

The starch was firmly held in the cells of *C. vulgaris*, being retained in the "holocellulose" fraction in association with the cell wall polysaccharides. Although cellulose has been reported as a constituent of certain *Chlorophyceae* (9, 18) there is no appreciable amount present in *C. vulgaris*, where the principal cell wall polysaccharide appears to be an alkali-soluble polyglucoside. Chitin is absent also, although it might be expected since it is found in certain fungi.

The autoclaving procedure which was necessary to extract the starch from *C. vulgaris*, probably caused some degradation. The isolated starch had iodophilic properties similar to Lintner soluble starch, a degraded wheat amylose sample having an average chain length of 60–80 units, and a wheat starch sample which had been subjected to the same treatment used to extract the starch from *C. vulgaris*. The disparity in the amylose contents obtained by the iodine staining and iodine titration methods shows that caution should be used in interpreting such data. The anomalies can be attributed to the relatively low molecular weight of chlorella amylose.

The starch synthesizing enzymes of *C. vulgaris* are essentially the same as those in higher plants. Glucose added to the medium is incorporated into starch without much breakdown and resynthesis. The synthesis of starch from glucose by this alga thus probably follows the course:

$$\text{Glucose} \rightarrow \text{glucose-1-phosphate} \xrightarrow{\text{phosphorylase}} \text{amylose} \xrightarrow{\text{Q-enzyme}} \text{amylopectin.}$$

This conclusion is further supported by the demonstration of an enzyme system in *Chlorella* which phosphorylates glucose (15).

The high rate of starch deposition in *C. vulgaris* cultured in a medium containing glucose made this study feasible. It might be argued that a different mechanism is used for formation of starch, in media devoid of sugars, where the carbohydrate is formed by photosynthesis. However there is no evidence to support such an argument, and it seems reasonable to suppose that the final stages of starch formation are the same whatever the source of the monosaccharide.

References

1. Abraham, S., Chaikoff, I. L., and Hassid, W. Z. J. Biol. Chem. 195 : 567. 1952.
2. Adams, G. A. and Castagne, A. E. Can. J. Research B, 26 : 325. 1948.
3. Allen, R. J. L. Biochem. J. (London), 34 : 858. 1940.
4. Bailey, J. M. Starch metabolising enzymes. Ph.D. Thesis, University of Wales. 1951. p. 138.

5. BAILEY, J. M., WHELAN, W. J., and PEAT, S. J. Chem. Soc. 3692. 1950.
6. BARKER, S. A. and BOURNE, E. J. Quart. Revs. (London), 7 : 56. 1953.
7. BATES, F. L., FRENCH, D., and RUNDLE, R. E. J. Am. Chem. Soc. 65 : 142. 1943.
8. BECKMAN, C. O. and ROGER, M. J. Biol. Chem. 190 : 467. 1951.
9. BLINKS, L. R. *In* Manual of phycology. *Edited by* G. M. SMITH. Chronica Botanica Company, Waltham, Mass. 1951. Chap. 14.
10. BOLD, H. C. *In* Manual of phycology. *Edited by* G. M. SMITH. Chronica Botanica Company, Waltham, Mass. 1951. Chap. 11.
11. BOURNE, E. J., HAWORTH, SIR N., MACEY, A., and PEAT, S. J. Chem. Soc. 924. 1948.
12. BROWN, A. H. Am. J. Botany, 33 : 228. 1946.
13. CALVIN, M., HEIDELBERGER, C., REID, J. C., TOLBERT, B. M., and YANKWICH, P. F. Isotopic Carbon. John Wiley & Sons, Inc., New York. 1948. p. 266.
14. ELSON, L. A. and MORGAN, W. T. J. Biochem. J. (London), 27 : 1824. 1933.
15. EMERSON, R. L., STAUFFER, J. F., and UMBREIT, W. W. Am. J. Botany, 31 : 107. 1944.
16. GREEN, D. E. and STUMPF, P. K. J. Biol. Chem. 142 : 355. 1942.
17. ISBELL, H. S., KARABINOS, J. K. V., FRUSH, H. L., HOLT, N. B., SCHWEBEL, A., and GALKOWSKI, T. T. J. Research Natl. Bur. Standards, 48 : 163. 1952.
18. IYENGAR, M. O. P. *In* Manual of phycology. *Edited by* G. M. SMITH. Chronica Botanica Company, Waltham, Mass. 1951. Chap. 3.
19. MCCREADY, R. M. and HASSID, W. Z. J. Am. Chem. Soc. 65 : 1154. 1943.
20. MEYER, K. H., FISCHER, E. H., STAUB, A., and BERNFELD, P. Helv. Chim. Acta, 31 : 2158. 1948.
21. MORRIS, D. L. Science, 107 : 254. 1948.
22. NEISH, A. C. Can. J. Botany, 29 : 68. 1951.
23. PARTRIDGE, S. M. Nature, 164 : 443. 1949.
24. PEAT, S., BOURNE, E. J., and BARKER, S. A. Nature, 161 : 127. 1948.
25. PEAT, S., PIRT, S. J., and WHELAN, W. J. J. Chem. Soc. 714. 1952.
26. PEAT, S., THOMAS, G. J., and WHELAN, W. J. J. Chem. Soc. 722. 1952.
27. PIRT, S. J., and WHELAN, W. J. J. Sci. Food Agr. 5 : 224. 1951.
28. SCHOCH, T. J. Cereal Chem. 18 : 121. 1941.
29. SHAFFER, P. A. and HARTMANN, A. F. J. Biol. Chem. 45 : 349. 1921.
30. SOWDEN, J. C. J. Am. Chem. Soc. 71 : 3568. 1949.
31. WHELAN, W. J., BAILEY, J. M., and ROBERTS, P. J. P. J. Chem. Soc. 1293. 1953.
32. WHISTLER, R. L. and DURSO, D. F. J. Am. Chem. Soc. 72 : 677. 1950.
33. WILSON, E. J., Jr., SCHOCH, T. J., and HUDSON, C. S. J. Am. Chem. Soc. 65 : 1380. 1943.

EFFECT OF INSULIN ON ETHANOL METABOLISM[1]

By E. J. Masoro[2] and Henry Abramovitch

Abstract

The role of insulin in ethanol metabolism was investigated with the aid of C^{14}-labeled ethanol. Surviving kidney and liver slices prepared from insulized rats oxidized ethanol to carbon dioxide at approximately the same rate as slices prepared from control rats. The data on the effect of insulin on the conversion of ethanol to lipids are equivocal since the *P* value is 0.036. There appears to be some increase in lipogenesis but not of an appreciable magnitude. The possibility that the beneficial effects noted in the treatment of acute alcohol intoxication with insulin may be the result of an increased synthetic metabolism is discussed.

Introduction

Although the ability of insulin to increase the rate of ethanol metabolism has not definitely been established, there can be little doubt that insulin is of value in the clinical treatment of acute alcoholism (21). Many clinicians feel that the therapeutic value of insulin is due to its ability to increase the rate of alcohol oxidation, thereby more quickly ridding the body of this substance.

A review of the literature shows that the great majority of workers who have investigated the problem have found that insulin was effective in accelerating the rate of metabolism of alcohol. The first of these workers was Supniewski (18) who found that the rate of alcohol disappearance from the blood of rabbits could be increased by administering insulin. Similar results have been reported in the case of the following species: rats (5), dogs (20), human beings (9, 10, 17). In 1941 Clark *et al.* (4) found that insulin accelerates the rate of ethanol disappearance from the blood and that pancreatectomy diminishes it. They also found that previous insulin injection increases the rate of ethanol utilization of liver slices of normal cats.

On the other hand, several investigators who also studied the disappearance of alcohol from the blood failed to show that insulin has an effect on ethanol metabolism (11, 12, 14). Mirsky and Nelson (16) concluded that a complete lack of insulin had no effect on ethanol metabolism since there was no impairment of alcohol utilization by the depancreatized dogs studied in their investigations.

In most of the investigations that have been undertaken, the disappearance of alcohol from the blood has been used as the criterion of ethanol oxidation. Since the rate of disappearance of alcohol from the blood may or may not parallel the rate of oxidation of alcohol to carbon dioxide, knowledge of ethanol metabolism from such data must be considered incomplete. In the present work C^{14}-ethanol was used as a tool to investigate the problem; this technique permits a study of the complete oxidation of the ethanol molecule as well as its conversion to lipids or any other synthetic product.

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Methods

Preparation of C^{14}-Ethanol

C^{14}-Ethanol was prepared by fermenting uniformly labeled C^{14}-glucose with baker's yeast, using the method of Bartlett and Barnet (1).

The C^{14} content of the ethanol was determined as described in an earlier paper (15).

Ethanol was measured by the colorimetric method of Gibson and Blotner (8).

Animals and Tissues

Liver and kidney tissues were chosen for *in vitro* studies since work reported in an earlier paper (15) established these tissues as the major sites of ethanol metabolism.

Rats of the Sprague-Dawley strain were used and were fed ad libitum for 10 days a high carbohydrate diet of the following composition: glucose, 62.3 gm.; vitamin mix, 0.7 gm.; choline, 0.3 gm.; casein, 22 gm.; Hubbel, Mendel, and Wakeman salt mix, 2.5 gm.; fat, 9 gm.; cod liver oil concentrate, 0.2 gm.; l-tocopherol, 0.003 gm.; and celluflour, 3.0 gm.

The vitamin mix is composed of the following measured in grams: 0.1 thiamine, 0.125 riboflavin, 0.5 calcium pantothenate, 0.1 pyridoxine, 0.4 nicotinic acid, 0.02 folic acid, 25 ascorbic acid, 40 inositol, 0.15 biotin, 0.4 para-aminobenzoic acid, 0.32 menadione, 213 celluflour. To every two kilograms of diet 15 γ of vitamin B_{12} were added.

The amount of food ingested during the last three days of life is recorded in Table I. On the sixth day 50% of the rats were placed on insulin treatment, each of them receiving eight units of protamine zinc insulin administered subcutaneously daily for three days. On the 10th day 20 units of unmodified insulin were injected subcutaneously and the rat was sacrificed one hour later.

Both the insulized and the control rats were killed by a blow on the head; the required tissues were rapidly excised and placed separately in Petri dishes containing ice-cold Krebs–Henseleit bicarbonate buffer solution (13). Tissue slices were prepared free-hand with a thin razor blade and each tissue was collected separately in a Petri dish containing the ice-cold buffer solution. Tissue slices were blotted free of excess buffer solution on a filter paper. Approximately 500 mgm. of tissue were weighed and then transferred to a specially designed incubation flask.

Incubation Procedure

The main compartment of the incubation flask contained about 500 mgm. of tissue slices, 4.5 ml. of Krebs–Henseleit bicarbonate buffer (13), and 0.5 ml. of C^{14}-ethanol solution. The concentration of ethanol in the medium was 24.2 mgm. %. The tissues were incubated for three hours at 37.5° C. Details of both the incubation procedure and the incubation flask are described by Chernick *et al.* (3).

Collection of $C^{14}O_2$

The $C^{14}O_2$ was collected using the method of Chernick *et al.* (3).

Isolation of Total Lipids

The total lipids were extracted from the tissue slices by the method of Felts *et al.* (7).

These extracted lipids were oxidized by means of Van Slyke and Folch wet combustion solution (19) and the liberated carbon dioxide collected as barium carbonate in 0.25 *N* barium hydroxide.

Determination of C^{14}

All substances were converted to barium carbonate for determination of the C^{14} content. The barium carbonate so formed was mounted and counted using a modification of the method of Enteman *et al.* (6).

Results

Liver slices from the noninsulized control rats converted 19–26% of the C^{14}-ethanol to $C^{14}O_2$ and 7–19% to C^{14}-lipids (Table I). The mean value is 22% for the $C^{14}O_2$ and 11% for the C^{14}-lipids. Not one animal showed a C^{14}-lipid synthesis greater than its $C^{14}O_2$ production.

Liver slices from the insulized rats converted 15–24% of the C^{14}-ethanol to $C^{14}O_2$ and 10–30% to C^{14}-lipids (Table I). The mean value for the $C^{14}O_2$ is

TABLE I

ETHANOL METABOLISM IN LIVERS FROM INSULIZED AND CONTROL RATS

Rat No.	Food intake*	Dosage of insulin (units)	Number of incubation flasks‡	Per cent of ethanol converted to	
				CO_2	Lipids
1 ♀	31	0	3	26	8
2 ♂	60	0	3	19	16
3 ♂	78	0	3	20	7
4 ♀	47	0	2	23	8
5 ♀	34	0	2	22	—
6	31	0	2	21	9
7 ♀	39	0	2	19	19
8 ♀	33	0	2	22	12
9 ♀	61	44†	3	18	29
10 ♀	67	44†	2	15	17
11 ♀	71	44†	2	20	30
12 ♀	62	44†	2	15	14
13 ♀	60	44†	2	24	10
14 ♀	71	44†	2	18	26
15 ♂	98	44†	3	20	11
16 ♂	76	44†	3	19	11

* *Grams of food ingested during the three days prior to sacrifice.*

† *Eight units of protamine zinc insulin daily for three days; 20 units of unmodified insulin one hour prior to killing.*

‡ *Each flask contained 500 mgm. of tissue slices, 4.5 ml. of Krebs–Henseleit bicarbonate buffer and 0.5 ml. of C^{14}-ethanol solution; the ethanol concentration was 24.2 mgm. %.*

18% while that of the C^{14}-lipids is 21%. It is interesting to note that in the case of one half of the insulized rats the C^{14}-lipid synthesis was greater than $C^{14}O_2$ production.

Kidney slices prepared from control rats oxidized 48 to 61% of C^{14}-ethanol to $C^{14}O_2$ while slices from the insulized rats oxidized 56 to 61% of the C^{14}-ethanol to $C^{14}O_2$ (Table II).

TABLE II

OXIDATIVE CAPACITY OF KIDNEY FROM INSULIZED AND CONTROL RATS

Rat No.	Dosage of insulin (units)	Number of incubation flasks†	% of ethanol converted to CO_2
5	0	1	48
7	0	1	54
8	0	1	61
11	44*	1	57
13	44*	1	56
14	44*	1	61

* See Footnote Table II.
† See Footnote Table II.

Discussion

The surviving slices of kidney and liver tissues prepared from insulized rats oxidized the C^{14}-ethanol in the incubation medium to $C^{14}O_2$ at approximately the same rate as the slices prepared from the control rats. Since the rate of ethanol oxidation in tissue slices from the major sites of alcohol metabolism (15), the kidney and the liver, is not influenced by insulin, it is highly improbable that insulin acts as a stimulant of ethanol oxidation in the intact rat. Yet the majority of investigators have found that insulin increases the rate at which alcohol disappears from the blood of a mammal (4, 5, 17, 18, 20) and from the incubation medium in the case of liver slices (4). It is possible that the increased disappearance of ethanol observed by earlier workers is due to the utilization of ethanol in a biosynthetic process. Since Chernick and Chaikoff (2) found that liver slices prepared from insulized rats converted C^{14}-glucose to fatty acids at 10 times the rate that slices from noninsulized animals did, it was felt than an increased lipogenesis from ethanol might well account for the higher rate of alcohol disappearance from the blood of insulized animals. However, the data obtained in the present study are equivocal, and although there seems to be some increase in the rate of lipogenesis from ethanol in the case of liver slices prepared from insulized rats, it is certainly not of the order of magnitude that would account for any appreciable increase of alcohol disappearance from the blood. Of course it is possible that some other synthetic process such as glycogenesis or a combination of synthetic pathways is accelerated by insulin and thus might increase the rate of alcohol disappearance to an extent to account adequately for clinical effects.

In any case, the results of the present study would make it appear that the beneficial effects of insulin in the treatment of acute alcohol intoxication are not due to the ability of insulin to increase the rate at which the body burns alcohol.

Acknowledgment

This investigation was supported by research grants from the Alcoholism Research Foundation and the National Research Council of Canada. The authors are indebted to Dr. G. Krotkov for providing C^{14}-labeled glucose.

References

1. Bartlett, G. R. and Barnet, H N. Quart. J. Studies Alc. 10 : 381. 1949.
2. Chernick, S. S. and Chaikoff, I. L. J. Biol. Chem. 186 : 535. 1950.
3. Chernick, S. S., Masoro, E. J., and Chaikoff, I. L. Proc. Soc. Exptl. Biol. Med. 73 : 348. 1950.
4. Clark, B. B., Morrissey, R., Fazekas, J. F., and Welch, C. S. Quart. J. Studies Alc. 1 : 663. 1941.
5. Dontcheff, L. Compt. rend. soc. biol. 130 : 1406. 1939.
6. Enteman, C., Lerner, S. R., Chaikoff, I. L., and Dauben, W. G. Proc. Soc. Exptl. Biol. Med. 70 : 364. 1949.
7. Felts, J. M., Chaikoff, I. L., and Osborn, M. J. J. Biol. Chem. 191 : 683. 1951.
8. Gibson, J. G. and Blotner, H. J. Biol. Chem. 126 : 551. 1938.
9. Goldfarb, W., Bowman, K. M., and Parker, S. J. Clin. Invest. 18 : 581. 1939.
10. Greenberg, L. A. Quart. J. Studies Alc. 3 : 347. 1942.
11. Gregory, R., Ewing, P. L., and Duff-White, V. Proc. Soc. Exptl. Biol. Med. 54 : 206. 1943.
12. Harger, R. W. and Hulpieu, H. R. J. Pharmacol. Exptl. Therap. 54 : 145. 1935.
13. Krebs, H. A. and Henseleit, Z. Hoppe-Seyler's Z. physiol. Chem. 210 : 33. 1932.
14. Loomis, T. A. Quart. J. Studies Alc. 11 ; 527. 1950.
15. Masoro, E. J., Abramovitch, H., and Birchard, J. R. Am. J. Physiol. 173 : 37. 1953.
16. Mirsky, A. and Nelson, N. Am. J. Physiol. 127 : 308. 1939.
17. Newman, H. W. and Cutting, W. C. J. Clin. Invest. 14 : 945. 1935.
18. Supniewski, J. V. J. Biol. Chem. 70 : 13. 1926.
19. Van Slyke, D. D. and Folch, J. J. Biol. Chem. 136 : 509. 1940.
20. Widmark, E. M. P. Biochem. Z. 282 : 79. 1935.
21. Wortis, J., Bowman, K. M., and Goldfarb, W. Med. Clin. N. Amer. 24 : 671. 1940.

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